

REVIEW

N-nitroso compounds and man: sources of exposure, endogenous formation and occurrence in body fluids

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Based on recent analytical data, total human exogenous exposure to *N*-nitrosamines is estimated to be 1.10 $\mu\text{mol/day}$; the major exposure sources are the diet (0.79 $\mu\text{mol/day}$, 80–120 $\mu\text{g/day}$; 72%), occupational exposure (0.15–0.30 $\mu\text{mol/day}$; 25%), cigarette smoking (0.02 $\mu\text{mol/day}$, 3.4 $\mu\text{g/day}$; 2%), and miscellaneous minor sources, including pharmaceutical products, cosmetics, indoor and outdoor air (0.001 $\mu\text{mol/day}$, 0.1 $\mu\text{g/day}$; 1%). Excretion of apparent total *N*-nitroso compounds (ATNC) in healthy adults is estimated to be $1.30 \pm 1.05 \mu\text{mol/day}$ in urine and between 1.56 ± 1.56 and $3.17 \pm 2.58 \mu\text{mol/day}$ in faeces. The excretion of volatile *N*-nitrosamines (*N*-nitrosodimethylamine), and *N*-nitrosamino acids and their derivatives (*N*-nitrososarcosine, *N*-nitrosoproline, *N*-nitrosothiazolidine-4-carboxylic acid and *N*-nitroso-2-methylthiazolidine-4-carboxylic acid) accounts for approximately 0.03% and 16.0% of urinary ATNC, respectively. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol and its *O*-glucuronide conjugate, two metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone present in urine of smokers, account for 0.2% of the urinary ATNC response; < 1.5% of the excretion of currently identified *N*-nitroso compounds in urine. The remaining *N*-nitroso compounds excreted in urine and those present in faeces are still unidentified. A crude mass balance between exogenous exposure and excretion in urine and faeces indicates that 45–75% of the total human exposure to *N*-nitroso compounds results through *in vivo* formation.

Key words: Biomonitoring, endogenous formation, exposure assessment, *N*-nitroso compounds.

Introduction

Prior to 1956, interest in *N*-nitroso compounds was limited to industrial applications such as the use of *N*-nitrosodimethylamine (NDMA) as a solvent in the vulcanization of rubber, and to the chemical laboratory. This situation changed rapidly after investigations of the hepatotoxicity suffered by workers in the rubber industry identified NDMA as inducing primary hepatic tumours in the rat (Magee and Barnes, 1956). Soon afterwards it became evident that *N*-nitroso compounds also occurred naturally in the environment (Ender *et al*, 1964), and

a large potential existed for their endogenous formation (Sander *et al*, 1968). As a consequence, concerns were raised over the possible health risks posed by these compounds.

In 1978, the American National Academy of Sciences concluded that the endogenous formation of *N*-nitroso compounds could be the largest source of exposure for the general population (National Academy of Sciences, 1978). Three years later in 1981, the major exogenous sources of human exposure to preformed *N*-nitroso compounds were

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assessed to be the diet (0.3–20 µg/day), cigarette smoking (17 µg/day), cosmetics (0.4 µg/day) and occupational exposure (10–180 µg/day) (National Academy of Sciences, 1981). Since these assessments, several, but not all *N*-nitrosamines, have been shown to induce a wide range of tumours in experimental animals (Preussmann and Steward, 1984; Lijinsky, 1992). Based on limited epidemiological evidence, the potential role of *N*-nitrosamines in the aetiology of human cancers has been speculated (Preston-Martin and Correa, 1989). Several reviews have reported the mechanisms by which *N*-nitroso compounds exert their biological effects (Archer, 1989; Shuker and Bartsch, 1994) and their relevance to human cancer (Magee, 1989; Bartsch, 1991; Hoffmann *et al.*, 1994; Reed, 1996).

Since 1981, the occurrence of preformed *N*-nitroso compounds in the human environment has been studied extensively (Tricker *et al.*, 1989a). Based on current literature data, the 1981 exposure assessment by the National Academy of Sciences (1981) has been re-evaluated, and the major sources of human exposure calculated. Mechanisms of endogenous nitrosation, methods for its quantification, and the concentrations of *N*-nitrosamines in human body fluids are presented. Special emphasis is given to the role of dietary habits, tobacco smoking, occupational and environmental exposure to preformed *N*-nitroso compounds, and the influence of certain disease states on the concentrations of *N*-nitroso compounds in human body fluids. Finally, the excretion of apparent total *N*-nitroso compounds (ATNC) in urine and faeces is compared with the current assessment of total human exposure in order to estimate the extent of endogenous nitrosation in man.

Formation of *N*-nitroso compounds

N-nitroso compounds are characterized by a nitroso group (--N=O) bonded to a nitrogen atom. The classical method for producing an *N*-nitrosamine is the reaction of a secondary amine with nitrous acid which is formed by the reaction of nitrite under acidic conditions (Ridd, 1961; Mirvish, 1975; Williams, 1983). Nitrite and nitrous acid *per se* are not nitrosating agents but are intermediates in the formation of the nitrosating species dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4) and the nitrous acidium ion ($\text{H}_2\text{O}^+\text{NO}$). The acidity of the aqueous medium determines the relative proportions of the nitrosating species (Ridd, 1961).

Nitrosation of the simplest aliphatic amine, methylamine, results in a complex mixture of products which

include NDMA; more complex primary aliphatic amines yield elimination, substitution and rearrangement products (Mende *et al.*, 1989).

Protonated secondary amines are unreactive towards N_2O_3 and nitrosation occurs typically at an optimum of pH 3.0–3.5. Introduction of ionizable groups near to the nitrosatable amino moiety, as in the case of α -amino acids, reduces the basicity of the amine and the pH maxima for nitrosation (Mirvish *et al.*, 1973). Depending on the amino acid residues present, nitrosation of dipeptides can produce *N*-nitrosodi-peptides (Tricker *et al.*, 1984) or low yields of *N*-nitrosoiminodialkanoic acids (Pollock, 1985). All peptides undergo nitrosation at the terminal primary amino group to yield a diazo-peptide, and at the peptide *N*-atoms to produce *N*-nitroso-peptides (Challis, 1989). Under acidic conditions diazo-peptides are usually formed more readily than *N*-nitroso-peptides, but quickly decompose. Under neutral conditions, diazo-peptides, once formed, are relatively stable. *N*-Nitroso-peptides are stable under both acid and neutral conditions. Peptides containing proline, cysteine, tyrosine, tryptophan, arginine, lysine, glutamine or asparagine residues undergo additional reactions with nitrosating agents: proline and tryptophan form specific *N*-nitroso derivatives, cysteine is oxidized via an *S*-nitroso intermediate to yield a disulphide (cystine) bridge, tyrosine forms a *C*-nitroso derivative, and arginine, lysine, glutamine and asparagine are subject to deamination reactions.

Simple symmetrical trialkylamines react slowly with nitrous acid; for more complex asymmetrical amines with substituents other than simple alkyl groups, no clear systematic approach can be taken to predict which alkyl substituent will be replaced by nitrosative dealkylation. As an example, nicotine reacts very slowly *in vitro* with nitrous acid in aqueous solution to produce 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanal (NNA), *N*-nitrosoornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hecht *et al.*, 1978), while alkaloids containing *N,N*-dialkyl side-chains and *N,N*-dialkylaromatic compounds are far more readily nitrosated (Loepky *et al.*, 1994).

Since acid catalysed nitrosation is inappreciable above about pH 5.0, *N*-nitrosamines in foodstuffs, consumer products and environmental media are most likely to be formed either by exposure to gaseous oxides of nitrogen (NO_x) or via an HCHO-catalysed reaction with nitrite (Keefer and Roller, 1973). Catalysis of nitrosation may be accelerated by thiocyanate and other anions (Fine and Tannenbaum, 1973). Conversely, virtually any

compound which reacts with nitrite can inhibit the formation of *N*-nitrosamines (Bartsch *et al.*, 1988). Ascorbic acid and ascorbate ions inhibit nitrosation by competing for the reaction with N_2O_3 , $\text{H}_2\text{O}^+\text{NO}$ and NO_x , in which ascorbic acid is oxidized irreversibly to form dehydroascorbic acid, and the nitrosating species is reduced to nitric oxide (NO) (Licht *et al.*, 1988a). Tocopherols react with nitrosating species in a manner analogous to ascorbic acid; the tocopherol is oxidized to the quinone while the nitrosating species is reduced to NO (Newmark and Mergens, 1981). Inhibition of proline, hydroxyproline and sarcosine nitrosation is particularly effective at pH 1.0–4.0, although this may not apply to other amines (Chang *et al.*, 1979). The formation of *N*-nitrosamines in consumer products, and current regulations to reduce their presence, has been reviewed (Challis, 1996).

Sources of exposure to *N*-nitroso compounds

Diet

Quantitatively the most abundant *N*-nitroso compounds present in the diet are non-volatile *N*-nitrosated amino acids and amino acid derivatives (NVNA). The major NVNA present in the diet are *N*-nitrososarcosine (NSAR), *N*-nitrosoproline (NPRO), *N*-nitroso-4-hydroxyproline (NHPRO), *N*-nitrosothiazolidine-4-carboxylic acid (NTCA), *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA) and *N*-nitroso-2-hydroxymethylthiazolidine-4-carboxylic acid (NHMTCA). Mechanisms for the formation and the concentrations of individual NVNA in different foodstuffs have been reviewed: these compounds occur mainly in smoked and nitrate-cured meats, dried and smoked fish, seafoods and smoked cheese at concentrations ranging between 10 and several thousand $\mu\text{g}/\text{kg}$ for individual NVNA (Tricker and Kubacki, 1992). Traditionally smoked foods such as Icelandic smoked mutton contain extremely high levels of NTCA (up to 6.76 mg/kg) in addition to other *N*-nitrosamines (Helgason *et al.*, 1984; Sen *et al.*, 1990). Cured and smoked meats may contain NVNA formed by condensation reactions of the amino acids serine and threonine with simple aldehydes or sugars, followed by nitrosation (Tricker and Kubacki, 1992). Similar condensation reactions involving tryptophan may explain the recent report of 13–346 $\mu\text{g}/\text{kg}$ 2-nitroso-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid in cured and smoked meats (Sen *et al.*, 1995). Although

the total daily exposure to NVNA has not been determined, estimates suggest an exogenous exposure of 10–100 $\mu\text{g}/\text{day}$ (Hotchkiss, 1989; Tricker and Preussmann, 1991a).

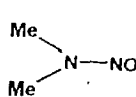
The major volatile *N*-nitrosamines (VNA) in the diet are NDMA, *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPPI) and *N*-nitrosothiazolidine (NTHz) (Tricker and Preussmann, 1991a). These compounds may be formed by the nitrosation of the corresponding precursor amines or thermal decarboxylation of NVNA during heat treatment of foods (Hotchkiss, 1989). Extensive dietary surveys performed in several different countries indicate that beer, cured meat and fish products contribute significantly to the total dietary exposure of 0.3–1.0 $\mu\text{g}/\text{day}$ VNA (Hotchkiss, 1989; Tricker and Preussmann, 1991a). Repeated investigations of beer (Tricker and Preussmann, 1991b; Sen *et al.*, 1996) and total diet surveys (Tricker *et al.*, 1991a) indicate a general decreasing trend in dietary exposure to VNA.

A limited range of foods contains additional *N*-nitroso compounds, albeit at low concentrations (Tricker and Preussmann, 1991a). One source for these compounds is the migration of preformed *N*-nitrosamines and nitrosatable precursor amines from direct-contact food packaging materials (Sen, 1988); in particular, elastic rubber nettings used for meat products (Pensabene and Fiddler, 1994). Similarly, treating apples with diphenylamine may result in contamination with trace levels of *N*-nitrosodiphenylamine (Lillard and Hotchkiss, 1994). Inadvertent contamination by these sources seldom exceeds 10 $\mu\text{g}/\text{kg}$ for individual *N*-nitroso compounds and makes only a negligible contribution to the total daily intake of *N*-nitrosamines. Of the 25 currently identified *N*-nitroso compounds in the diet, the major preformed *N*-nitrosamines are shown in Figure 1.

Tobacco and tobacco smoke

Tobacco-specific *N*-nitrosamines (TSNA) are formed during the curing and processing of tobacco by nitrosation of *Nicotiana* alkaloids (Figure 2). NNA has not been identified in tobacco or tobacco smoke, probably due to the reactivity of the aldehyde group, which could be either oxidized to yield 4-(methylnitrosamino)-4-(3-pyridyl) butyric acid (iso-NNAC) or reduced to yield 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) (Hoffmann *et al.*, 1994). Reduction of the keto group in NNK yields 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNN is primarily formed by

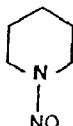
Volatile N-nitrosamines



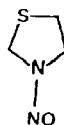
NDMA



NPYR

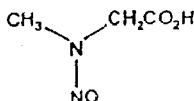


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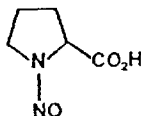


NTHz

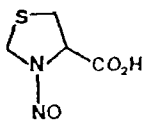
Non-volatile N-nitrosamino acids and derivatives



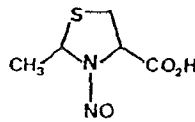
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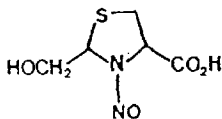
NPRO



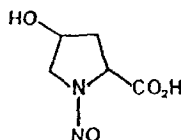
NTCA



NMTCA



NHMTCA



NHPRO

Figure 1. Major preformed N-nitroso compounds in the diet. Abbreviations used for the N-nitrosoamines are: NDMA, N-nitrosodimethylamine; NPYR, N-nitrosopyrrolidine; NPIP, N-nitrosopiperidine; NTHz, N-nitrosothiazolidine; NSAR, N-nitrososarcosine; NPRO, N-nitrosoproline; NTCA, N-nitrosothiazolidine-4-carboxylic acid; NMTCA, N-nitroso-2-methylthiazolidine-4-carboxylic acid; NHMTCA, N-nitroso-2-hydroxymethylthiazolidine-4-carboxylic acid; NHPRO, N-nitrosohydroxyproline.

nitrosation of the tobacco alkaloid nor nicotine (Mirvish *et al.*, 1977), and to a lesser extent, by nitrosation of nicotine-N-oxide (Klimisch and Stadler, 1976). Microbial degradation of nicotine-N-oxide to pseudoxy nicotine (Maeda *et al.*, 1978), which is rapidly and irreversibly nitrosated to NNK (Caldwell *et al.*, 1993), may be the major route of NNK

formation in tobacco. Anabasine and anatabine are nitrosated to yield N-nitrosoanabasine (NAB) and N-nitrosoanatabine (NAT), respectively.

Commercial filter cigarettes have mean mainstream smoke deliveries of 75 ± 41 (range 19–135) ng NNN, 52 ± 29 (range 21–110) ng NNK, 73 ± 47 (range 18–155) ng NAB/NAT, 7.8 ± 2.9 (range

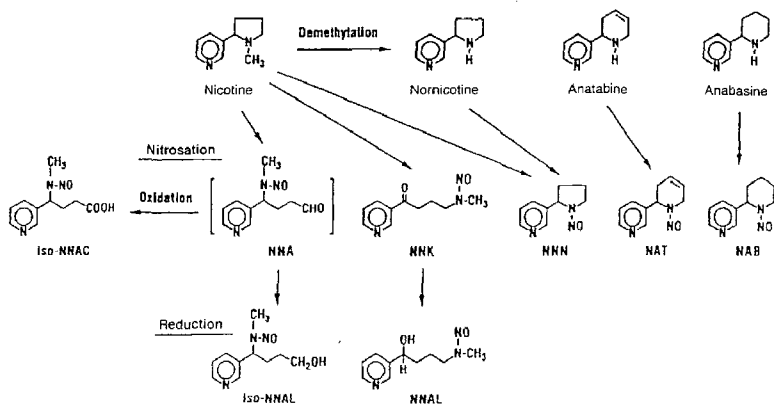


Figure 2. Formation of tobacco-specific *N*-nitrosoamines. Abbreviations used for the *N*-nitrosoamines are: NNA, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanal; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NAB, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid.

5.8–15.2) ng NDMA, 2.4 ± 1.4 (range <1.0–3.9) ng *N*-nitrosoethylmethanamine (NEMA) and 9.0 ± 3.0 (range 3.9–15.2) ng NPYR per cigarette (Tricker *et al.* 1991b). The presence of <11 ng/cigarette iso-NNAL (Tricker *et al.* 1991b) and <5 ng/cigarette iso-NNAC (Djordjevic *et al.* 1991; Tricker *et al.* 1993) in mainstream cigarette smoke have been less seldomly reported. Cigarette smoke does not contain NPRO (Brunnemann *et al.* 1983), and no evidence exists to show that other NVNA are present in tobacco smoke. TSNA are primarily particle-bound, while VNA occur in the gas phase (Adams *et al.* 1987). Assuming 70% retention of particle-bound TSNA in the lung during oral inhalation (Hinds *et al.* 1983), a smoker of 20 filter cigarettes per day would have a total exposure of about 3.0 µg/day TSNA (1.05 ± 0.57 µg NNN, 0.73 ± 0.41 µg NNK and 1.02 ± 0.66 µg NAB/NAT) and about 0.4 µg/day VNA.

Since TSNA and VNA are also transferred to sidestream cigarette smoke (Adams *et al.* 1987), and presumably exhaled by smokers, trace levels occur in environmental tobacco smoke (ETS) present in indoor air (Brunnemann *et al.* 1992; Klus *et al.* 1992; Tricker *et al.* 1994). Extensive smoking under poor ventilation conditions results in mean ETS concentrations of 2.8 ± 1.6 (range ND–6.0) ng/m³ NNN,

4.9 ± 9.6 (range ND–13.5) ng/m³ NNK, 19.8 ± 11.4 (range 7.9–45.0) ng/m³ NDMA and 10.0 ± 6.6 (range 3.5–27.0) ng/m³ NPYR (Klus *et al.* 1992). NDMA has also been reported in indoor air of discotheques, bars and trains (Brunnemann and Hoffmann, 1978). Smoking within the home increases the mean background NDMA concentration from 9.5 ± 0.8 (range 8.6–10.4) to 12.5 ± 6.0 (range 3.3–25.7) ng/m³ (Tricker *et al.* 1994). The occurrence of NDMA in homes occupied by non-smokers shows that ETS is not the only source of NDMA in indoor air. Both NDMA and NPYR are volatilized in fumes during cooking (Sen *et al.* 1976), and other combustion sources may contribute to NDMA found in indoor air (Brunnemann and Hoffmann, 1978). Assuming an adult has a daily respiratory volume of 20 m³ and that 10–15% deposition of particle-bound TSNA occurs in the lung during nasal inhalation (Hiller *et al.* 1982), non-smoker exposure to TSNA present in ETS in a poorly ventilated office (Klus *et al.* 1992) can be estimated to be 0.4 ± 0.2 (range ND–0.8) ng/h NNN and 0.6 ± 0.4 (range ND–1.7) ng/h NNK. Therefore, over an 8 h working day exposure is unlikely to exceed more than 20 ng TSNA.

Smokeless tobacco contains seven TSNA (Hoffmann *et al.* 1994), at least 10 NVNA including

NSAR, NPRO, *N*-nitrosoazetidine-4-carboxylic acid (NAZCA) and 3-(*N*-nitrosomethylamino)propionic acid (NMPA) (Djordjevic *et al.* 1989; Tricker and Preussmann, 1991c), and various VNA (Tricker and Preussmann, 1991c). The use of smokeless tobacco is difficult to quantify, and exposure estimates range between 27.5 µg/day TSNA for users of nasal snuff tobacco (Tricker and Preussmann, 1991c) to 11.5 mg/day NNK for toombak snuff-dippers in Sudan (Idris *et al.* 1991). The presence of TSNA in the saliva of toombak users during chewing ranges from 0.7 to 30.6 µg/ml (Idris *et al.* 1992), 10- to 100-fold higher than the levels found in saliva of users of other types of smokeless tobacco (Hoffmann and Adams, 1981; Sipahimalani *et al.* 1984; Nair *et al.* 1985; Bhude *et al.* 1986; Brunnemann *et al.* 1987; Österdahl and Storch, 1988).

Cosmetic products

During the 1970s, cosmetic products frequently contained simple VNA such as NDMA and *N*-nitrosomorpholine (NMOR) (Spiegelhalter and Preussmann, 1984) and long-chain *N*-nitroso-*N*-methylalkylamines such as *N*-nitroso-*N*-methyldecylamine and *N*-nitroso-*N*-methyltetradecylamine (Hecht *et al.* 1982). By the beginning of the 1990s most European cosmetics were free from these contaminants (Kamp and Eisenbrand, 1991) while approximately 15% of personal care products still contained 12–235 µg/kg *N*-nitrosodiethanolamine (NDELA) and/or 40–215 µg/kg *N*-nitrosobis-(2-hydroxypropyl)amine (NDHPA) (Eisenbrand *et al.* 1991). American cosmetic products still contained 210–2,960 µg/kg NDELA, and sunscreens were reported to contain 780–5,050 µg/kg 2-ethylhexyl-4-(*N*-methylnitrosamino)benzoate (NPABAO) (Havery and Chow, 1994). Rapid photodegradation of NPABAO in sunscreen products by ultraviolet light (Havery and Chow, 1994) probably limits the extent of human exposure (Kenney *et al.* 1995); however, the photolytic decomposition products have not been identified.

With the exception of some American products, negligible exposure to *N*-nitrosodialkanolamines such as NDELA and NDHPA is predicted to occur through normal use of personal care cosmetics (European Chemical Industry Ecology and Toxicology Center, 1990). Most contemporary European personal care products with the exception of some shampoos, night creams and lipsticks containing < 50 µg/kg *N*-nitrosamines (Challis *et al.* 1995a). Changes in process chemistry and inclusion of antioxidants and iminium ion traps in cosmetic

formulations are expected to result in further reductions in *N*-nitrosamine formation and contamination in the near future (Challis *et al.* 1995b).

Pharmaceutical products

In the 1970s several prescription and over-the-counter pharmaceutical products were reported to contain various VNA, in particular NDMA (Krull *et al.* 1979). More recent studies indicate considerable reductions in contamination by VNA (Dawson and Lawrence, 1987a), so that pharmaceutical products can, in general, be disregarded as a source of exogenous *N*-nitrosamine exposure. However, *N*-mononitrosopiperazine (MNPz) contamination of products containing piperazine (Dawson and Lawrence, 1987b) may still be a current problem (see following section on pharmaceutical products). Nicotine-containing chewing gum used as a smoking cessation aid has been reported to contain ≤ 380 ng TSNA/mg gum (Österdahl, 1990). Other nicotine-containing pharmaceutical products available prior to 1992 also contained trace levels of TSNA (about 20–40 ng/g nicotine), but most contemporary products now appear to be free of contamination (Tricker, unpublished results).

Ambient air

Concentrations of 10–40 ng/m³ NDMA, NDEA and NMOR have been measured in ambient air in a heavily polluted industrial area of Austria (Spiegelhalter and Preussmann, 1987). Similar levels have been reported in Germany with the highest outdoor air concentrations (10–90 ng/m³) occurring in the vicinity of chemical plants (Akkan *et al.* 1991). Higher levels of 9–220 ng/m³ NDMA and 11–220 ng/m³ NDEA have been reported in ambient air of industrialized areas in the former USSR (Ianysheva *et al.* 1991). More recent data report 50–600 ng/m³ VNA in Moscow city air with the highest concentrations found near to industrial areas (Khesina *et al.* 1996).

Occupation

During the 1970s, widespread ambient exposure to various *N*-nitrosamines also occurred in the leather industry (< 0.05–47 µg/m³ NDMA and < 0.05–2 µg/m³ NMOR); the rubber industry (0.02–5.5 µg/m³ NDMA, 0.13–1.4 µg/m³ *N*-nitrosodiethylamine (NDEA), 0.01–3.9 µg/m³ NPYR, 0.02–250 µg/m³ NMOR and 0.01–1,230 µg/m³ *N*-nitrosodiphenylamine (NDPhA)); the surfactant industry (0.03–0.8 µg/m³ NDMA); and metal foundries (0.02–1.4 µg/m³ NDEA) (Rounbehler and

Fajen, 1982). In several cases, worker exposure to individual VNA in the rubber industry probably exceeded 50 $\mu\text{g}/\text{day}$ (Spiegelhalder, 1984). Synthetic cutting fluids based on aqueous solutions of secondary and tertiary alkanolamines with added nitrite salts as corrosion inhibitors used in commercial metal-working operations contained 200–30,000 mg/kg (0.02–3% by weight) NDELA (Fan *et al.*, 1977) and up to 30,000 mg/kg *N*-nitroso-5-methyl-1,3-oxazolidine (Stephany *et al.*, 1978).

Reformulation of synthetic cutting fluids and discontinued use of nitrite salts as corrosion inhibitors have generally reduced contamination during storage to < 50 mg/kg NDELA (Keefer *et al.*, 1990). In a recent German survey of metal workshops, concentrated cutting fluids contained a mean of 20.6 ± 5.0 (range 2–135) mg/l NDELA, and their use resulted in mean ambient air concentrations of 214 ± 391 (range < 50–1,000) ng/m^3 NDELA (Fuchs *et al.*, 1995). In another recent Canadian survey of 14 metal-working factories, diluted cutting fluids contained 0.93 ± 1.96 (range 0.01–7.53) mg/l NDELA and their use resulted in airborne levels of 40.8 ± 60.2 (range ND–193) ng/m^3 NDELA (Fadlallah *et al.*, 1996). The same metal-working factories also contained ambient levels of 34.4 ± 29.1 (range ND–102) ng/m^3 NDMA; $31\text{--}167$ ng/m^3 NDMA was detected in four factories and 25 ng/m^3 NDEA in one factory. No correlations were observed between the NDELA concentration in cutting fluids and the levels of *N*-nitrosamines in the corresponding factory air. Contamination of diluted cutting fluids may be caused by nitrite formed by bacterial reduction of nitrate in water used for dilution and/or absorption of atmospheric NO_x . The use of bismorpholinomethane as a preserving agent may be the source of 0.02–5.0 mg/l NMOR recently found in some used diluted cutting fluids (Eisenbrand *et al.*, 1996). Similar to the situation with cosmetics containing alkanolamines, inclusion of antioxidants and iminium ion traps may have the potential to reduce *N*-nitrosamine formation both during storage and use in metal-working operations.

Recent area air monitoring in a German factory manufacturing rubber seals and components for automobiles determined time-weighted average concentrations of 30–120 ng/m^3 NDMA and 29–78 ng/m^3 *N*-nitrosodibenzylamine (NDBzA) (Dietrich *et al.*, 1996). Concurrent to this, a survey performed by the National Institute of Occupational Safety and Health (NIOSH) measured time-weighted average concentrations of 19.2 ± 28.6 (range 2.3–88.5) $\mu\text{g}/\text{m}^3$ NDMA, 0.25 ± 0.4 (range ND–1.03) $\mu\text{g}/\text{m}^3$ NDEA,

3.89 ± 2.77 (range 1.4–10.2) $\mu\text{g}/\text{m}^3$ NPPI, 0.11 ± 0.05 (range 0.05–0.16) $\mu\text{g}/\text{m}^3$ NPYR and 0.26 ± 0.20 (range 0.06–0.55) $\mu\text{g}/\text{m}^3$ NMOR in a US factory producing rubber vehicle seals (Rey and Fajen, 1996). Total VNA concentrations averaged 23.72 ± 31.38 (range 4.50–99.5) $\mu\text{g}/\text{m}^3$. Personal breathing zone air samples collected for 28 workers showed exposure to 3.16 ± 2.47 (range 0.47–11.4) $\mu\text{g}/\text{m}^3$ NDMA, 0.17 ± 0.19 (range 0.01–0.81) $\mu\text{g}/\text{m}^3$ NDEA, 1.67 ± 0.91 (range 0.20–4.39) $\mu\text{g}/\text{m}^3$ NMOR. The average exposure to total VNA in breathing zone air samples was 5.25 ± 3.41 (range 0.76–16.3) $\mu\text{g}/\text{m}^3$. VNA concentrations in area and personal breathing zone samples were related to different manufacturing processes and job categories, respectively. The highest VNA concentrations appeared to occur at production lines producing rubber using *N,N*-dinitrosopentamethylene tetramine as a vulcanization agent. Although there are no numerical occupational *N*-nitrosamine standards in the US, the exposures measured are much higher than the German standard of 1 $\mu\text{g}/\text{m}^3$ total *N*-nitrosamines in general industry and 2.5 $\mu\text{g}/\text{m}^3$ total *N*-nitrosamines for certain processes, such as rubber vulcanization. In Germany, changes in production technology, control and avoidance of raw materials either containing *N*-nitrosamines (eg *N,N*-dinitrosopentamethylene tetramine) or susceptible to nitrosation have reduced occupational exposure to total *N*-nitrosamines to < 0.1 $\mu\text{g}/\text{kg}$ body weight/day (Wolf, 1989). In the absence of such measures, exceedingly higher exposure of production workers to *N*-nitrosamines still occurs, as is evident from the NIOSH study (Rey and Fajen, 1996) and recent data from the former USSR showing mean winter levels of 244 ± 75 $\mu\text{g}/\text{m}^3$ NDMA and 836 ± 407 $\mu\text{g}/\text{m}^3$ NDEA in some areas of rubber production, with a mean cumulative NDMA and NDEA exposure of 63 and 1,064 $\mu\text{g}/\text{day}$ in summer and winter, respectively (Solionova *et al.*, 1992).

Endogenous nitrosation

There is no reason to doubt that amine substrates yield similar nitrosation products endogenously to those formed under *in vitro* conditions by classical nitrosation mechanisms. The acidic gastric compartment would be expected to be the major site of endogenous nitrosation in man. In biological systems, the reactivity and relative stability of *N*- and *S*-containing compounds favours the formation of *N*-nitrosamines and *S*-nitrosothiols compared to alternative *C*- and *O*-nitroso products.

NPOR is formed *in vivo* by the nitrosation of proline (Ohshima and Bartsch, 1981). NTCA and

NMTCA are both formed *in vivo* by condensation of cysteine with formaldehyde or acetaldehyde, respectively, followed by nitrosation (Tricker and Preussmann, 1987). The yields of NTCA and NMTCA are about 250–500 and 60–300 times greater than that of NPRO at pH 1.0–4.0, respectively; optimal formation of NTCA and NMTCA occurs at pH 2.5 and 4.5, respectively, compared to pH 2.5 for NPRO (Ohshima *et al.*, 1984). Condensation of homocysteine with formaldehyde and subsequent nitrosation probably results in the formation of *N*-nitrosotetrahydro-4*H*-1,3-thiazine-4-carboxylic acid (NHTTCA) (Nair *et al.*, 1986). The secondary amines dimethylamine, pyrrolidine and piperidine, precursors to NDMA, NPYR and NP1P, respectively, are present in nearly all body fluids (Tricker *et al.*, 1992). *S*-Nitrosation of thiols, including cysteine and homocysteine (O'Neill and Ohshima, 1987; Stamler *et al.*, 1992), and formation of NTCA (Tsuda and Kurashima, 1991a) may provide protection against formation of endogenous *N*-nitrosamines.

Vitamin C (ascorbic acid) and vitamin E (α -tocopherol) both inhibit *in vivo* nitrosation when administered with a nitrosatable amine substrate (Ohshima and Bartsch, 1981; Wagner *et al.*, 1985). However, in most commercial forms of vitamin E, the tocopherol is esterified and incapable of inhibiting *N*-nitrosation, while the effect of ascorbic acid is only temporal, because of its rapid excretion in urine (Helser *et al.*, 1991; Mirvish *et al.*, 1995). Only negligible inhibition of NPRO formation occurs under normal dietary conditions after daily supplementation with a 500 mg ascorbic acid tablet, and a capsule containing 100 mg ascorbic acid and 100 IU α -tocopherol each day before breakfast, lunch, dinner and before retiring for the night (Garland *et al.*, 1986). This is not totally surprising, since maximal endogenous nitrosation in the gastric compartment appears to occur 1–2 h after meals (Mirvish *et al.*, 1995) at which time gastric ascorbate levels are almost depleted. Endogenous formation of *N*-nitrosamines may occur at sites other than the gastric compartment and via different pathways not involving acid-catalysed mechanisms (Hill, 1996).

Cell-mediated nitrosation

Many different mammalian cells produce NO via a common biochemical pathway involving oxidation of the terminal guanido-nitrogen of L-arginine to citrulline by NO synthase (NOS) in the presence of O₂ and NADPH (Förstermann *et al.*, 1991; Stuehr *et al.*, 1991). A major role for endogenous NO

production has been described in virtually every vertebrate organ system involving functions as diverse as bioregulation of vascular tone, platelet aggregation, central and autonomic neurotransmission, immune regulation, bacterial killing, control of airway reactivity and basal airway tone (Ånggård, 1994). NOS isoforms are generally classified as either Ca²⁺ dependent and 'constitutive' (cNOS) or Ca²⁺ independent and 'inducible' (iNOS) (Stuehr *et al.*, 1991). The Ca²⁺-mediated calmodulin binding of cNOS isoforms can be stimulated by mediators, including bradykinin, acetylcholine, calcium ionophore, histamine, leukotrienes and platelet activating factor. Isoforms of iNOS are induced by interferon- α , endotoxin, lipopolysaccharide (LPS), tumour necrosis factor (TNF- β), interleukin-1 and a variety of cytokines.

The biological effects of endogenous NO formation depend on a complex interaction of different conditions, including the rate of NO production and its rate of diffusion, the concentrations of potential reactants and necessary enzymes, and the distance between generator and target cells. The half-life of NO in biological systems is estimated to be 0.1 s (Kelm and Schrader, 1990) and is inversely proportional to its concentration (Wink *et al.*, 1993). NO can interact directly with cellular guanylate cyclase for signalling (Palmer *et al.*, 1987), oxygen species (eg superoxide) to form reactive intermediates resembling hydroxyl radicals (Ischiropoulos *et al.*, 1992), or molecular oxygen to form nitrosating species (Iyengar *et al.*, 1987; Cooney *et al.*, 1992). Reactions with water ultimately yield nitrite and nitrate.

Total endogenous cell-mediated mammalian nitrate production under normal conditions amounts to about 1 mmol/day or 1 mg/kg body weight/day in man (Green *et al.*, 1981). On a body weight basis, the same order of magnitude is also found in experimental animals (Leaf *et al.*, 1990). Intense physical activity (Leaf *et al.*, 1990; Persson *et al.*, 1993) and immunostimulation during nonspecific intestinal diarrhoea and fever (Hegesh and Shiloah, 1982) are probably the most important inducers of increased endogenous NO synthesis in otherwise healthy individuals. Immunostimulated NO synthesis occurs primarily in macrophages resulting in the generation of cellular microenvironmental concentrations of NO at a rate of 1 mM/min for several hours (Ischiropoulos *et al.*, 1992). Although the production of NO occurs after a lag phase (approximately 4–6 h after stimulation), the amount of NO generated is generally higher than that formed by constitutive processes. The low rates of endogenous NO

production by constitutive processes and activated cells may consequently result in a highly efficient generation of nitrosating species (Tamir *et al.*, 1993).

Stimulated macrophages readily nitrosate aliphatic and heterocyclic secondary amines *in vitro* after addition to the medium at physiological pH; the yield of *N*-nitrosamines being partially dependent on amine lipophilicity, and inversely related to basicity (Miwa *et al.*, 1987). Stimulated macrophages also nitrosate proline and thiazolidine-4-carboxylic acid (TCA) to yield NPRO and NTCA, respectively (Miwa *et al.*, 1989). Addition of nitrite and amines to non-stimulated cells produces negligible yields of *N*-nitrosamines. Compounds generally capable of inhibiting acid-catalysed nitrosation by competing for nitrosating species inhibit nitrosation by macrophages, although ascorbic acid shows a complex behaviour in which nitrosation is enhanced at low ascorbate concentrations (5–50 μM) and inhibited at higher concentrations (Kosaka *et al.*, 1989). Inflammatory neutrophil-mediated nitrosation is also inhibited by ascorbic acid (Grisham *et al.*, 1992), antioxidants and immunosuppressive agents (Grisham and Miles, 1994).

In rats administered both morpholine and arginine, immunostimulation by treatment with *Escherichia coli* LPS significantly increases excretion of nitrate and *N*-nitroso-(2-hydroxyethyl)glycine (NHEG), a metabolite of endogenously formed NMOR (Leaf *et al.*, 1991). Significant formation of NTCA from administered TCA occurs after stimulation of ascorbic acid-deficient mutant rats with *E. coli* LPS (Kosaka *et al.*, 1990) and in rats with acute hepatic injury caused by *Propionibacterium acnes* (Wu *et al.*, 1993). In the absence of administration of nitrosatable amines, elevated formation of nitrate and NDMA occurs in LPS-stimulated woodchucks with chronic woodchuck hepatitis infection (Lui *et al.*, 1991).

Bacterial nitrosation

Several bacterial strains, both denitrifying and non-denitrifying, of the genera *Alcaligenes*, *Bacillus*, *Escherichia*, *Klebsiella*, *Neisseria*, *Proteus* and *Pseudomonas* catalyse the nitrosation of secondary amines (Calmels *et al.*, 1985, 1988; O'Donnell *et al.*, 1988; Mackerness *et al.*, 1989; Charrière *et al.*, 1991). Nitrosating activity is linked to the presence of nitrate reductase genes (Calmels *et al.*, 1987, 1988; Ralt *et al.*, 1988); however, expression of these genes is not always associated with nitrosating activity (Charrière *et al.*, 1991). Enterobacteriaceae and Pseudomonadaceae generally express both nitrate

reductase and nitrosating activities (Calmels *et al.*, 1985; Leach *et al.*, 1987; O'Donnell *et al.*, 1988; Charrière *et al.*, 1991); however, not all strains within the same genus or even the same species catalyse nitrosation (Calmels *et al.*, 1985). Nitrosation also depends on the physiological state of the bacteria: *E. coli* catalyses nitrosation of various secondary amines in the resting phase (Calmels *et al.*, 1985), while some enteric bacteria (e.g. *Pseudomonas stutzeri*) catalyse nitrosation only in the growth phase (Mills and Alexander, 1976). Denitrifying bacteria, when suitably induced, show a consistently greater *in vitro* nitrosating capacity than non-denitrifying bacteria (Leach *et al.*, 1987). In both denitrifying and non-denitrifying bacteria cytochrome *cd*₁-nitrite reductase appears to catalyse nitrosation through the production of NO or NO⁺-like species (Calmels *et al.*, 1996).

Bacterial nitrosation at neutral pH is inhibited by ascorbic acid (Mackerness *et al.*, 1989) and cysteine (O'Donnell *et al.*, 1988), suggesting that the nitrosating species are most likely N₂O₂ or N₂O₃ produced via an NO intermediate (Ji and Hollocher, 1988); however, the exact mechanism remains unknown (Zumft, 1993). Catalysis of nitrosation follows classical Michaelis-Menton kinetics and does not fit simple first- and second-order kinetics observed for chemical nitrosation of secondary amines (Calmels *et al.*, 1985). The specific nitrosation rate is inversely related to the pK_a of the amine substrate (Calmels *et al.*, 1985); high nitrite concentrations (Calmels *et al.*, 1985; Leach *et al.*, 1987) and decreasing pH (O'Donnell *et al.*, 1988) result in inhibition.

Ambient nitrogen oxides

Inhalation of atmospheric NO_x in particular NO₂, may increase both endogenous nitrate and nitrite exposure as well as the pool of endogenous nitrosating species dissolved in airway lining fluid. Weakly acidic conditions in the airway (Lopez-Vidriero *et al.*, 1977) are likely to alter the distribution of dissolved NO_x to favour decomposition of nitrite to the nitrosating species N₂O₂ (Ridd, 1978). Dissolved NO₂ present in the lung can react to form nitrous acid by hydrogen abstraction from unsaturated fatty acids, with the initiation of lipid peroxidation (Pryor and Lightsey, 1981), or interact with cholesterol in lung tissues and blood to form cholesteryl-3- β -nitrite, a lipid soluble nitrosating species (Kobayashi and Kubota, 1980).

In the absence of elevated ambient NO_x concentrations, pulmonary epithelial cells, macrophages,

neutrophils, mast cells, autonomic neurons, vascular smooth muscle cells, fibroblasts and endothelial cells are all potentially capable of generating NO in the airways via the NOS pathway (Gaston *et al.* 1994), as is evident from the observation that expired air normally contains NO of endogenous origin (Persson *et al.* 1993). The formation of S-nitrosothiols in airway lining fluid (Gaston *et al.* 1993) may be an important mechanism mitigating the toxicity derived from reactions of NO_x in the lung (Stamler *et al.* 1992).

Animal studies provide only limited evidence that inhalation of NO_x can result in endogenous N-nitrosamine formation. Gavage of rodents with morpholine followed by exposure to 20–50 ppm NO₂ does not result in significant NMOR formation (Mirvish *et al.* 1981; Van Stee *et al.* 1983), or has produced artifact results during analysis (Iqbal *et al.* 1980). Similarly, NMOR produced in *ex vivo* studies with isolated rat lungs perfused with 10 mM morpholine and ventilated with 20 ppm NO₂ (Postlethwait and Mostafa, 1983) appears to be caused by artifact formation occurring in the lung perfusion apparatus (Cooney *et al.* 1986b). NDMA formation has been reported in rats and rabbits pretreated with aminopyrine, a readily nitrosatable pharmaceutical, and exposed to 100 ppm NO₂ (Uozumi *et al.* 1982). In a more recent study in which mice were given morpholine by gavage and exposed to 20 ppm ¹⁵NO₂, endogenous formation of both ¹⁴NMOR and ¹⁵NMOR occurred, suggesting that both ambient ¹⁵NO₂ can be converted endogenously to a nitrosating species resulting in the formation of ¹⁵NMOR and that ¹⁴NMOR is produced via an ongoing mammalian process (Van Stee *et al.* 1995).

Biomonitoring of N-nitrosamino acids in urine

The major NVNA found in human urine are NSAR, NPRO, NTCA and NMTCa (Ohshima *et al.* 1984; Tricker and Preussmann, 1987). Excretion of NMPA (Nair *et al.* 1986; Forman *et al.* 1988; Zatonski *et al.* 1989), NHPRO (Tricker and Preussmann, 1987), NACa (Nair *et al.* 1986; Zatonski *et al.* 1989) and NHTCA (Nair *et al.* 1986) has also been reported. Most human biomonitoring studies have compared NSAR, NPRO, NTCA and NMTCa excretion in populations from low- and high-prevalence areas for specific cancers, or healthy control subjects to clinical patient collectives (Table 1). Only those studies are presented in which 24-h urine samples have been collected and excretion calculated as µg/day NVNA.

When available, data for the excretion of nitrate is also presented.

Animal studies indicate that almost quantitative urinary excretion (>95%) occurs for NPRO (Chu and Magee, 1981; Ohshima *et al.* 1982), NTCA and NMTCa (Ohshima *et al.* 1984) within 24 h of oral administration, with only trace levels occurring in faeces. NSAR is primarily excreted in urine ($87.7 \pm 2.4\%$) with only about 0.07% excretion in faeces, while NHPRO is excreted almost quantitatively in both urine ($46.8 \pm 2.2\%$) and faeces ($46.2 \pm 3.2\%$) (Ohshima *et al.* 1982). Excretion studies have not been reported for other minor NVNA (NMPA, NACa and NHTCA) found in urine.

NSAR and NPRO are chemically stable and easily amenable to gas chromatographic analysis using a thermal energy analyser (Massey, 1988). Depending on the solvent system used, NTCA and NMTCa may be subject to acid-catalysed decomposition during extraction from urine (Lu *et al.* 1986) and thermal degradation may occur upon analysis by gas chromatography. Quantitation may also be impeded by the failure to achieve adequate baseline resolution of NVNA during gas chromatographic separation (Ohshima *et al.* 1984; Tsuda *et al.* 1986). Collaborative studies have not been published to compare interlaboratory variation in the determination of NVNA in urine; therefore, caution must be taken in any cross-study comparisons.

Intraindividual and interindividual variations in urinary NPRO excretion have been studied in 24 h urine samples collected four times per week for 5 weeks from 24 healthy volunteers (Garland *et al.* 1986). During the last 3 weeks of the study, each subject received daily supplementation with a 500 mg ascorbic acid tablet, a capsule containing 100 mg ascorbic acid and 100 IU α-tocopherol each day before breakfast, lunch, dinner and before retiring for the night. The mean NPRO excretion for all subjects was 3.31 ± 4.06 µg/day in the absence of vitamin supplementation and 3.22 ± 4.00 µg/day during supplementation. Urinary NPRO excretion ranged from <0.20 to 38.1 µg/day (480 samples) for all subjects. The mean urinary excretion of NPRO (20 measurements per subjects) in different individuals ranged from 0.76 ± 0.63 to 9.04 ± 7.41 µg/day. The mean relative standard deviations for interindividual and intraindividual differences were 116% and 84%, respectively. The interindividual variation ranged between 36% and 253%. Similar results were also reported for NTCA excretion which showed a highly significant correlation ($R \approx 0.74$) with NPRO excretion when the means for each individual were

Table 1. Daily urinary excretion of nitrate and *N*-nitrosamino acids in population studies^{a,b}

Population and comments	n	Nitrate (mg/day)	NSAR (µg/day)	NPRO (µg/day)	NTCA (µg/day)	NMTCA (µg/day)	Total (µg/day)	Reference
(West) Germany								
General population	20	97.1 ± 48.1 (20-218)	0.42 ± 0.37 (ND-1.2)	2.23 ± 0.51 (1.4-3.1)	7.24 ± 4.24 (2.3-16.7)	5.08 ± 2.81 (3.6-14.9)	14.43 ± 5.48 (8.9-28.5)	Tricker <i>et al</i> , 1989b
General population	24	62.9 ± 29.8 (29.8-105.5)	0.94 ± 0.78 (ND-2.6)	2.84 ± 1.06 (1.2-5.2)	12.04 ± 5.87 (5.0-29.0)	2.84 ± 1.63 (ND-6.2)	18.66 ± 7.84 (8.5-37.2)	Tricker and Eisenbrand, unpublished results
United Kingdom								
Hospital staff	10	89.1 ± 37.4 (30-117)	0.36 ± 0.32 (ND-2.4)	2.15 ± 0.32 (1.6-2.7)	6.92 ± 2.99 (3.9-12.3)	4.68 ± 0.98 (3.5-6.7)	14.07 ± 3.68 (9.7-20.9)	Tricker, 1996
Paraplegic patients	30	47.0 ± 33.4 (1.6-145.9)	0.48 ± 0.36 (ND-2.6)	3.24 ± 0.48** (1.9-5.5)	9.32 ± 3.86 (3.2-27.5)	6.02 ± 2.52 (2.8-10.4)	25.16 ± 14.82* (13.5-68.4)	
Poland								
Low gastric cancer prevalence	47	90 (7-344)	0.10* (0.1-1.2)	2.0 (0.4-19.3)	7.5 (0.1-80.3)	-	11.1 ^c (1.5-109)	Zatonski <i>et al</i> , 1989
High gastric cancer prevalence	50	96 (36-475)	0.24 (ND-2.4)	1.8 (ND-23.4)	10.5 (ND-23.2)	-	14.6 ^c (0.3-243.3)	
Egypt								
Healthy population	27	139.3 ± 82.2 (35-402)	3.40 ± 5.91 (ND-29.6)	7.16 ± 5.07 (1.2-20.1)	11.69 ± 7.60 (3.5-33.4)	9.08 ± 5.55 (3.1-23.2)	31.20 ± 22.07 (9.7-94.8)	Tricker <i>et al</i> , 1989b
<i>Schistosomiasis</i> (bilharzia) infection	27	143.6 ± 136.3 (16-506)	6.01 ± 3.45 (ND-13.4)	17.01 ± 7.15**d (2.9-33.6)	24.37 ± 9.27**d (3.7-41.4)	15.12 ± 6.81 (1.8-29.2)	62.91 ± 21.96**d (13.2-108.7)	
Bilharzial bladder cancer	23	175.0 ± 190 (35-855)	6.24 ± 9.99 (2.1-9.9)	12.61 ± 3.37 (2.7-17.2)	15.93 ± 3.78 (9.4-23.7)	9.47 ± 4.60 (4.9-24.1)	44.94 ± 7.31 (24.6-65.5)	
Japan								
General population (men)	19	-	-	1.6 ± 1.1 (0.6-4.5)	6.4 ± 5.5 (0.7-22.9)	5.4 ± 3.6 (0.4-14.5)	13.4 ± 7.2 (2.0-30.9)	Tsuda <i>et al</i> , 1987
General population (women)	14	-	-	1.9 ± 0.9 (0.8-3.9)	12.1 ± 5.9*** (3.0-20.7)	10.2 ± 7.9** (1.5-24.7)	24.1 ± 11.4 (5.8-40.6)	
Seventh Day Adventists (men)	18	-	-	4.3 ± 6.0 (0.3-24.4)	5.1 ± 3.4 (1.2-11.7)	3.6 ± 2.9 (0.4-11.7)	13.1 ± 10.8 (2.3-47.2)	
Seventh Day Adventists (women)	18	-	-	3.0 ± 3.2 (0.2-12.6)	5.2 ± 4.8 (0.6-19.4)	3.4 ± 3.7 (0.4-13.8)	11.6 ± 10.0 (1.4-34.2)	
Low gastric cancer prevalence	52	95 [73-117]	-	3.8 [2.6-5.0]	12.4 [7.7-17.1]	3.2 [2.1-4.3]	20.2 [14.7-26.6]	Kamiyama <i>et al</i> , 1987
High gastric cancer prevalence	52	145*** [118-176]	-	6.1 [4.1-8.1]	5.7* [2.0-9.4]	2.8 [1.2-4.4]	14.8 [7.4-22.2]	

Table 1 (continued)

Population and comments	<i>n</i>	Nitrate (mg/day)	NSAR (µg/day)	NPRO (µg/day)	NTCA (µg/day)	NMTCA (µg/day)	Total (µg/day)	Reference
Low oesophageal cancer prevalence (men)	29	203 ± 111		4.9 ± 4.3	10.3 ± 8.7	5.0 ± 5.6	–	Tsugane <i>et al.</i> , 1992
Low oesophageal cancer prevalence (women)	28	151 ± 98	–	3.3 ± 3.1	6.6 ± 5.4	1.6 ± 2.0	–	
High oesophageal cancer prevalence (men)	28	167 ± 99	–	3.5 ± 2.3	17.3 ± 15.6	12.3 ± 13.8	–	
High oesophageal cancer prevalence (women)	26	218 ± 82	–	4.3 ± 3.1	18.9 ± 15.5	7.5 ± 6.1	–	
Very high oesophageal cancer prevalence (men)	37	166 ± 100	–	4.4 ± 5.5	19.1 ± 21.1	10.0 ± 11.7	–	
Very high oesophageal cancer prevalence (women)	24	187 ± 133	–	5.9 ± 14.3	17.1 ± 19.2	7.3 ± 5.0	–	
China								
Low oesophageal cancer prevalence	40	48 [37–61]	0.1 [0.1–0.18]	1.7 [0.9–2.9]	1.9 [1.6–4.2]	0.1 [0.1–0.94]	5.6 [3.6–8.1]	Lu <i>et al.</i> , 1986
High oesophageal cancer prevalence	44	94*** [69–119]	0.49** [0.17–0.63]	5.7*** [4.1–7.7]	13.7*** [8.6–16.9]	0.69 [0.1–0.98]	21.2*** [14.2–25.3]	
Low oesophageal cancer prevalence	44	–	1.5 ± 3.2	2.0 ± 2.6	0.3 ± 1.6	1.3 ± 3.6	6.3 ± 7.2 ^f	Lu <i>et al.</i> , 1987
High oesophageal cancer prevalence	44	–	–	5.7 ± 8.2	2.6 ± 4.9	–	8.7 ± 11.2 ^f	
	48	–	2.6 ± 5.7	5.9 ± 9.2	1.9 ± 6.1	0.1 ± 0.8	12.4 ± 16.5 ^f	
	54	–	3.8 ± 8.9	4.2 ± 6.3	4.5 ± 6.5	1.0 ± 4.8	21.3 ± 19.9 ^f	
	57	–	–	6.6 ± 11.2	2.6 ± 4.3	–	13.7 ± 13.7 ^f	
	50	–	1.6 ± 8.5	4.4 ± 6.2	6.7 ± 12.1	0.8 ± 3.8	13.6 ± 18.1 ^f	

Abbreviations: NSAR, *N*-nitrososarcosine; NPRO, *N*-nitrosoproline; NTCA, *N*-nitrosothiazoline-4-carboxylic acid; NMTCA, *N*-nitroso-2-methylthiazolidine-4-carboxylic acid; ND, not detected.

^aReported data excludes studies on smokers and nonsmokers (see Table 2). Data reported as either arithmetic means ± SD (range) or geometric means [95% confidence limits].

^bAll tests of significance compare areas of low- and high-prevalence for specific cancers unless otherwise stated: **P* > 0.05, ***P* < 0.01, ****P* < 0.001.

^cTotal includes 4-(*N*-nitrosomethylamino)propionic acid (NMPA) and NMTCA.

^dSignificance compared to normal population.

^eSignificance compares men to women.

^fTotal includes an unidentified compound (presumed to be NMPA).

considered (Tannenbaum, 1986). Other studies report similar interindividual variations in NSAR, NPRO, NTCA and NMTCa excretion in single subjects (Ohshima *et al.* 1984; Tsuda *et al.* 1986; Tricker and Preussmann, 1987). The influence of sex on NVNA excretion has not been subject to thorough investigation, although some evidence exists to suggest that a sex-related difference in NTCA and NMTCa excretion occurs, with higher excretion rates in women (Tsugane *et al.* 1992). These studies suggest that multiple measurements are required to assess the actual excretion status of an individual.

Effect of diet

The lowest mean excretion of NPRO (about 0.3 $\mu\text{g/day}$) has been reported in Indian subjects (Nair *et al.* 1985, 1986; Chakradeo *et al.* 1994); considerably lower than that reported in other populations (Table 1). The low level of NPRO excretion may be a result of the mainly vegetarian Indian diet, which lacks nitrate-preserved foodstuffs. A low NPRO excretion is observed in Canadian vegetarians ($0.80 \pm 0.93 \mu\text{g/day}$) and lacto-vegetarians ($1.37 \pm 1.49 \mu\text{g/day}$) (Stich *et al.* 1984). Swedish lacto-vegetarians also have a low NPRO excretion ($1.33 \pm 0.94 \mu\text{g/day}$) compared with subjects eating meat-containing diets ($2.30 \pm 2.12 \mu\text{g/day}$) (Halling *et al.* 1989). Changing from eating meat-containing diets to vegetarian diets results in significant sequential daily reductions in NPRO excretion (Stich *et al.* 1984).

Canadian Inuits consuming 'western' diets containing nitrate-preserved foods have significantly increased excretion of NPRO ($P < 0.05$) compared to Inuits with traditional fresh meat and fish diets (Stich and Hornby, 1988). Consumption of nitrate-cured meat products is also associated with increased urinary NPRO excretion in Denmark (Møller *et al.* 1989). More than 90% of NPRO in meat products is protein-bound and not amenable to analysis without prior hydrolytic or enzymatic digestion (Dunn and Stich, 1984; Sen *et al.* 1989), consumption of which provides a significant source of urinary NPRO both in man (Stich *et al.* 1984) and experimental animals (Perciballi *et al.* 1989; Sen *et al.* 1989). In ferrets, protein-bound NPRO in diets containing cured meat accounts for about 70% of urinary NPRO excretion, while <17% is formed endogenously (Perciballi *et al.* 1989).

Dietary fish significantly increases NTCA excretion (Tsuda *et al.* 1988); consumption of Tara-chiri, a traditional Japanese dish containing cod and radish, significantly increases ($P < 0.001$) NTCA excretion

from 7.9 ± 4.2 to $110 \pm 64.5 \mu\text{g/day}$. Other cod-based diets may increase NTCA excretion to as much as $300 \mu\text{g/12 h}$. The cause of the increased excretion of NTCA appears to be due to endogenous nitrosation of relatively high concentrations of TCA (about 4.0 mg/kg) present in fish.

Other dietary factors have not been extensively studied in individual subjects or populations. The effects of diet on the urinary excretion of NPRO and NTCA suggest that in the absence of a modest degree of dietary control, comparisons of NVNA excretion in small population groups may be significantly influenced by a small number of individuals with dietary habits deviating from the majority of the population group.

Effect of nitrate

Dietary supplementation with 217 mg [^{15}N]-nitrate results in a 5- to 6-fold increase in NPRO and NTCA excretion; only about 10-60% of urinary NPRO contains the ^{15}N label and the incorporation of [^{15}N]-nitrate into NPRO, but not NTCA, is inhibited by both ascorbic acid and α -tocopherol (Wagner *et al.* 1985). Higher levels of nitrate supplementation (600 mg/day) significantly increases urinary excretion of NSAR (0.43 ± 0.37 vs $0.98 \pm 0.57 \mu\text{g/day}$; $P < 0.05$), NPRO (2.09 ± 0.60 vs $15.3 \pm 5.9 \mu\text{g/day}$; $P < 0.05$), NTCA (4.93 ± 3.01 vs $34.8 \pm 28.8 \mu\text{g/day}$; $P < 0.05$), NMTCa (6.83 ± 3.48 vs $77.0 \pm 56.6 \mu\text{g/day}$; $P < 0.05$), NHPRO (1.44 ± 0.45 vs $16.2 \pm 9.2 \mu\text{g/day}$; $P < 0.05$) and total NVNA excretion (15.4 ± 5.9 vs $159.8 \pm 84.6 \mu\text{g/day}$; $P < 0.05$) (Tricker and Preussmann, 1987). After intravenous administration of 5.56-8.35 g/day nitrate as NH_4NO_3 to healthy adult volunteers, maximum urinary NPRO excretion increases to $320 \mu\text{g/l}$ (Ellen and Schuller, 1984).

Under normal dietary conditions, a strong correlation ($P = 0.002$) is reported between intake of nitrate in drinking water and urinary NPRO excretion in rural Nebraska (Mirvish *et al.* 1992). Similarly, nitrate intake from drinking water is strongly associated with NPRO excretion in rural Danish non-smokers (χ^2 test for trend; $P = 0.08$), but not in smokers (Møller *et al.* 1989). A significant correlation between urinary nitrate and NPRO excretion ($R = 0.297$; $P = 0.0001$) has also been reported for Colombians living in a high-prevalence area for gastric cancer (Stillwell *et al.* 1991a). Nonsignificant but positive trends between urinary nitrate and NPRO excretion are found in both Italian (Knight *et al.* 1992) and Polish (Zatonski *et al.* 1989) subjects from different areas with

contrasting rates of gastric cancer. Contradictory results show both a correlation (Tsugane *et al.* 1992) or no correlation (Kamiyama *et al.* 1987) between urinary nitrate and NPRO excretion in Japanese populations living in high-prevalence areas for gastric cancer. Chinese studies show significant correlations between urinary nitrate and NPRO excretion in subjects ($R = 0.47$; $P = 0.0001$) living in low- and high-prevalence areas for nasopharyngeal cancer in southern China (Zeng *et al.* 1993) and in subjects ($R = 0.39$; $P < 0.01$) living in low- and high-prevalence areas for oesophageal cancer in northern China (Lu *et al.* 1986).

Effect of smoking

Mechanisms by which smoking may influence NVNA excretion have been reviewed (Tsuda and Kurashima, 1991b). Cigarette smoke contains NO_x (mainly in the form of NO (Williams, 1980; Norman *et al.* 1983)), formaldehyde and acetaldehyde (Houlgate *et al.* 1989). It is speculated that condensation of these aldehydes, which are present in tobacco smoke, with cysteine in airway lining fluid produces nitrosatable precursors to NTCA and NMTCa; however, studies in which mainstream cigarette smoke is passed into phosphate buffer (pH 7.4) containing cysteine indicate that only negligible amounts of NMTCa are formed (Tsuda and Kurashima, 1991b). Cigarette smoke also contains hydrogen cyanide (Norman *et al.* 1983) and acrylonitrile (Grob, 1962), both of which are metabolized to thiocyanate (Fiedler and Wood, 1956; Lambotte-Vandepaer *et al.* 1985), an effective catalyst of nitrosation *in vitro* (Fine and Tannenbaum, 1973) and *in vivo* in experimental animals (Ohshima *et al.* 1982; Licht *et al.* 1988b).

Several studies have compared the daily excretion of NVNA in smokers and nonsmokers (Table 2). Under experimentally controlled dietary conditions smoking significantly increases ($P < 0.05$) NPRO excretion (Hoffmann and Brunnemann, 1983); however, when dietary restrictions are used to prevent ingestion of significant levels of preformed NPRO and inhibitors of endogenous NPRO formation, only a slight, but not significant, increase is observed in smokers (Ladd *et al.* 1984). In a controlled diet study in which a male volunteer smoked 20 cigarettes/day for 20 nonconsecutive days and did not smoke for 20 nonconsecutive days during a 4-month period, increased urinary excretion of NPRO ($P < 0.01$), NTCA ($P < 0.001$), NMTCa ($P < 0.01$) and total NVNA ($P < 0.001$) occurred during the smoking period (Tsuda *et al.*

1986); however, the increase was not significantly different to that observed during consumption of an unrestricted diet (Tsuda *et al.* 1987). Urinary excretion of NTCA and NMTCa was also reported to be significantly higher in male smokers compared with both female smokers and male nonsmokers; the influence of sex on NTCA and NMTCa excretion was more significant than that of smoking. According to an Italian study, smoking does not affect the urinary excretion of NPRO; however, total NVNA (NSAR, NPRO, NTCA and NMTCa) excretion significantly increased in smokers of blond tobacco ($P < 0.05$) and black tobacco ($P < 0.05$), but not in smokers of both types of tobacco, when compared with nonsmokers (Malaveille *et al.* 1989). After correction for urinary creatinine, statistically significant positive dose-effect relationships were observed between urinary nicotine plus cotinine with NPRO ($P < 0.05$) and total NVNA ($P < 0.01$) excretion (Malaveille *et al.* 1989). Other studies have reported no significant effect of smoking on the excretion of NVNA (Ohshima *et al.* 1984; Cooney *et al.* 1986a; Nair *et al.* 1986; Knight *et al.* 1991; You *et al.* 1996).

In population studies performed in low- and high-prevalence areas for gastric cancer in which smoking was also taken into consideration, its effect on urinary NVNA excretion is even less clear. In agreement with the above Italian study (Malaveille *et al.* 1989), smoking had no effect on urinary NPRO excretion in two separate Italian studies (Knight *et al.* 1991, 1992). No significant effect of smoking was found on NPRO excretion in Polish subjects; however, smokers had a tendency for higher excretion of NMTCa and NMTCa with increasing number of cigarettes smoked (Zatonski *et al.* 1989). In Japanese subjects, no effect of smoking was found on NPRO and NMTCa excretion while a significant increase ($P < 0.005$) in NTCA excretion was observed (Kamiyama *et al.* 1987). Contrary to all these observations, NPRO excretion is lower in Colombian smokers compared with nonsmokers (Stillwell *et al.* 1991b).

Contradictory results have been reported on the effect of smoking in four Chinese population studies performed by the same working group. No significant effect of smoking was observed on urinary NSAR, NPRO, NTCA, NMTCa and total NVNA excretion in two representative subgroups of 50 current smokers and 33 nonsmokers from a study population of 538 subjects (Chang-Claude *et al.* 1991), or in all of 4,000 subjects (Wu *et al.* 1993), from a high-prevalence area for oesophageal cancer.

Table 2. Daily urinary excretion of nitrate and N-nitrosamino acids in healthy nonsmokers and smokers^{a,b}

Population and comments	n	Nitrate (mg/day)	NSAR (µg/day)	NPRO (µg/day)	NTCA (µg/day)	NMTCA (µg/day)	Total (µg/day)	Reference
(West) Germany								
Nonsmokers	10	93.7 ± 45.4 (41-195)	0.39 ± 0.34 (ND-1.0)	2.09 ± 0.44 (1.4-2.6)	5.79 ± 3.45 (3.3-12.6)	5.03 ± 1.53 (3.6-8.6)	13.33 ± 4.64 (8.9-24.4)	Tricker <i>et al.</i> , 1989
Smokers	10	106.8 ± 54.8 (30-218)	0.52 ± 0.44 (ND-1.2)	2.28 ± 0.57 (1.4-3.1)	9.07 ± 4.09 (4.8-16.7)	7.22 ± 3.46 (4.2-14.9)	19.09 ± 6.49 (12.0-28.5)	
France								
Nonsmokers	18	-	0.27 ± 0.31 (ND-0.09)	4.12 ± 4.52 (0.9-14.3)	5.93 ± 5.91 (0.9-21.4)	3.09 ± 3.57 (0.4-10.5)	13.45 ± 11.84 (3.9-34.4)	Ohshima <i>et al.</i> , 1984
Smokers	8	-	0.54 ± 0.58 (ND-1.6)	4.36 ± 3.71 (1.1-12.4)	14.49 ± 13.34 (3.1-35.0)	7.41 ± 6.10 (1.0-19.8)	26.91 ± 18.95 ^c (7.7-53.8)	
Nonsmokers	7	-	0.27 ± 0.34 (ND-0.9)	4.49 ± 4.21 (0.9-10.5)	8.56 ± 7.00 (1.7-21.4)	2.43 ± 3.60 (0.6-10.5)	16.09 ± 11.84 (3.9-34.4)	Nair <i>et al.</i> , 1986
Smokers	7	-	0.73 ± 1.08 (ND-3.0)	5.87 ± 3.65 (1.6-12.4)	17.29 ± 13.12 (3.1-35.0)	6.27 ± 6.34 (1.0-19.8)	30.89 ± 18.88 (7.2-53.8)	
Italy								
Nonsmokers	32	75.50 ± 45.77 (19-173)	-	2.19 ± 1.18 (0.5-5.4)	-	-	10.59 ± 6.17 ^c (1.7-26.3)	Malaveille <i>et al.</i> , 1989
Smokers of blond tobacco	48	89.92 ± 50.82 (18-232)	-	2.48 ± 1.29 (0.5-5.5)	-	-	14.99 ± 10.49 ^{c,e} (2.6-51.0)	
Smokers of black tobacco	15	79.73 ± 26.35 (27-134)	-	2.99 ± 1.79 (0.7-5.8)	-	-	16.85 ± 11.01 ^{c,e} (1.9-39.5)	
Smokers of mixed tobacco	9	69.44 ± 41.49 (33-167)	-	1.57 ± 0.82 (0.6-3.4)	-	-	10.09 ± 7.05 ^c (4.6-27.4)	
All smokers	72	85.24 ± 45.75 (21-232)	-	2.48 ± 1.42 (0.5-5.8)	-	-	14.76 ± 10.30 ^{c,e} (1.9-51.0)	

Table 2. (Continued)

Population and comment	<i>n</i>	Nitrate (mg/day)	NSAR (µg/day)	NPRO (µg/day)	NTCA (µg/day)	NMTCA (µg/day)	Total (µg/day)	Reference
Japan								
Nonsmoking men	19	—	—	1.6 ± 1.1 (0.6–4.5)	6.4 ± 5.5 (0.7–22.9)	5.4 ± 3.6 (0.4–14.5)	13.4 ± 7.2 (2.0–30.9)	Tsuda <i>et al.</i> , 1987
Nonsmoking women	20	—	—	1.9 ± 0.9 (0.8–3.9)	12.1 ± 5.9** ^a (3.0–20.7)	10.2 ± 7.9* ^a (1.5–24.7)	24.1 ± 11.4 (5.8–40.6)	
Smoking men	17	—	—	2.2 ± 1.2 (0.8–3.9)	10.5 ± 6.5* ^a (3.6–23.1)	8.8 ± 4.1* ^a (1.4–14.9)	24.2 ± 11.4 (5.8–40.6)	
Abstinent male smoker on normal diet	14	—	—	1.4 ± 0.8 (0.7–3.6)	7.9 ± 6.4*** ^c (2.2–24.9)	8.7 ± 6.4*** ^c (1.1–27.6)	18.0 ± 12.4 (4.6–48.9)	Tsuda <i>et al.</i> , 1986, 1987
Abstinent male smoker on 20 day fixed diet	20	—	—	1.1 ± 0.5 (0.3–2.6)	3.9 ± 1.1 (1.8–6.0)	5.6 ± 1.9 (2.4–9.2)	10.6 ± 2.8 (5.9–14.7)	
Male smoker on 20 day fixed diet	20	—	—	1.8 ± 0.9*** ^c (0.4–4.1)	8.7 ± 4.6*** ^c (2.7–21.3)	8.5 ± 4.1*** ^c (2.9–19.0)	19.0 ± 6.5*** ^c (9.1–28.5)	

Abbreviations: NSAR, *N*-nitrososarcosine; NPRO, *N*-nitrosoproline; NTCA, *N*-nitrosothiazoline-4-carboxylic acid; NMTCA, *N*-nitroso-2-methylthiazolidine-4-carboxylic acid; ND, not detected.

^aReported data excludes population studies in areas with low- and high-prevalence for specific cancer (see Table 1). Data reported as arithmetic means ± SD.

^bAll tests of significance compare nonsmokers to smokers unless otherwise stated: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

^cTotal includes NSAR, NTCA and NMTCA.

^dSignificance compared to nonsmoking men

^eSignificance compared to abstinent male smoker on fixed diet.

In two smaller studies, smokers had a tendency for higher excretion of NPRO, NTCA, NMTCa and total NVNA ($P < 0.01$) in low- and high-prevalence areas for oesophageal cancer in northern China (Lu *et al.* 1986), and significantly higher excretion of NPRO ($P < 0.01$), NTCA ($P < 0.01$) and total NVNA ($P < 0.001$) than nonsmokers in low- and high-prevalence areas for nasopharyngeal cancer in southern China (Zeng *et al.* 1993).

Effect of infection

Infection with *Opisthorchis viverrini* (liver fluke) in northeast Thailand is claimed to increase basal NPRO excretion from 4.80 ± 0.80 to 12.37 ± 3.44 $\mu\text{g/day}$ (Srianujata *et al.* 1987). Although hepatic *O. viverrini* infection results in stimulation of NOS (Srivatanakul *et al.* 1991), some doubt exists as to whether the higher NPRO levels observed in infected subjects is a result of an increased potential for endogenous nitrosation (Haswell-Elkins *et al.* 1994). *Schistosomiasis* infection (bilharzia) in Egypt significantly increases NVNA excretion (Tricker *et al.* 1989b). Bacterial infection of the urinary bladder increases urinary excretion of NPRO (2.25 ± 3.64 vs 0.94 ± 0.81 $\mu\text{g/l}$), NTCA (8.26 ± 20.22 vs 1.91 ± 3.14 $\mu\text{g/l}$) and NMTCa (1.64 ± 7.66 vs 0.25 ± 0.74 $\mu\text{g/l}$), and significantly increases nitrite excretion from nondetected levels to 2.16 ± 3.63 (range ND–15.2) mg/l (Ohshima *et al.* 1987). Bacterial infection of the bladder is nearly always associated with an increase in NVNA excretion and urinary excretion of nitrite. Paraplegic patients with bacterial infection of the bladder have significantly ($P < 0.05$) increased excretion of total NVNA compared with healthy subjects (Tricker. 1996).

Urinary diversion in which the bladder is resected and the ureters implanted into the sigmoid colon (ureterosigmoidostomy) results in direct contact of urinary amines and nitrate with the faecal bacterial flora. In comparison to normal bladder urines, rectal urine samples from ureterosigmoidostomy patients contain significantly less nitrate (58.7 ± 24.2 vs 16.7 ± 14.3 mg/l ; $P < 0.0001$), more nitrite (ND vs 1.35 ± 1.68 mg/l ; $P < 0.0001$), significantly higher levels of NSAR (0.93 ± 0.92 vs 0.27 ± 0.28 $\mu\text{g/l}$; $P < 0.02$), NPRO (3.11 ± 2.63 vs 1.40 ± 0.45 $\mu\text{g/l}$; $P < 0.001$) and NTCA (6.51 ± 4.49 vs 4.04 ± 2.65 $\mu\text{g/l}$; $P < 0.05$), and significantly less NMTCa (2.45 ± 3.07 vs 3.55 ± 3.28 $\mu\text{g/l}$; $P < 0.05$) (Tricker *et al.* 1989c). Daily administration of 2 g ascorbic acid (1 g in children) does not significantly reduce NVNA and nitrite excretion in ureterosigmoidostomy patients (Käble *et al.* 1991).

Biomonitoring of volatile N-nitrosamines in urine

A mean basal excretion of 34.8 ± 28.8 (range < 5 –190) ng/day NDMA has been reported in 24 healthy subjects studied four times per week for 5 weeks (Garland *et al.* 1986). NDMA excretion correlated significantly ($R = 0.69$; $P < 0.005$) with atmospheric NO_2 levels. The mean intraindividual urinary excretion (20 measurements per subject) ranged from 22.4 ± 10.9 to 75.4 ± 35.0 ng/day NDMA, and was not affected by daily supplementation with 900 mg vitamin C and 400 IU vitamin E during the last 2 weeks of the study. The mean relative standard deviation for interindividual and intraindividual differences were 52% and 51%, respectively. On an individual basis, the intraindividual variation ranged between 37% and 86%. The mean urinary excretion of 24.6 ± 14.0 pg/ml NDMA (480 24 h urine samples) agrees well with that of 25.8 ± 16.4 (range ND–63) pg/ml NDMA found in a smaller study (Webb *et al.* 1979).

The very low levels of NDMA excretion in healthy subjects is supported by animal studies in which hepatic first-pass metabolism of low doses of NDMA precludes significant excretion in urine (Swann *et al.* 1984; Anderson *et al.* 1992). In the rat, oral doses of 50 or 350 μg NDMA/rat results in only 0.02 or 0.03% excretion in urine, respectively (Spiegelhalder *et al.* 1982). VNA present in the urinary bladder may be metabolized by urothelial cells (Airoldi *et al.* 1992) or absorbed into the blood stream, resulting in increased systemic exposure (Hawksworth and Hill. 1974). As a consequence, determination of urinary VNA does not reflect exogenous exposure or endogenous formation.

Effect of alcohol and smoking

Basal NDMA excretion is significantly increased in nonsmoking drinkers (41.3 ± 22.2 ng/day ; $P < 0.01$) and smoking drinkers (65.0 ± 31.6 ng/day ; $P < 0.001$) compared with subjects who do not drink or smoke (32.2 ± 21.8 ng/day) (Cooney *et al.* 1986a). Smoking alone significantly increases ($P < 0.0001$) NDMA excretion to 53.3 ± 26.8 ng/day . Experimental studies show that consumption of 500 ml beer containing 12–30 μg NDMA results in 0.5–1.3% excretion of NDMA in urine; when the same NDMA dose was administered in orange juice, excretion of NDMA was only observed after fortification of orange juice with 6% ethanol (Spiegelhalder *et al.* 1982). Other studies have failed to detect NDMA excretion in fasting subjects after

consumption of alcohol (Milligan *et al.* 1986). However, the limit of detection in both studies (2.5 ng/ml NDMA) would not have been sensitive enough to detect the basal NDMA excretion reported in other studies (Webb *et al.* 1979; Cooney *et al.* 1986a; Garland *et al.* 1986).

Effect of infection

Endogenous nitrosation caused by bacterial infection of the urinary tract can result in excretion of 0.94 ± 2.42 (range ND–11.84) $\mu\text{g/l}$ NDMA, 0.5 ± 1.4 (range ND–6.2) $\mu\text{g/l}$ NPYR and 0.17 ± 0.35 (range ND–1.68) $\mu\text{g/l}$ NPIP (Ohshima *et al.* 1987). In spine-injured paraplegic patients with bacterial infection of the urinary tract, mean daily excretion of 0.65 ± 0.69 (range ND–2.68) $\mu\text{g/day}$ NDMA, 0.39 ± 0.50 (range ND–1.96) $\mu\text{g/day}$ NPYR, 0.25 ± 0.44 (range ND–2.13) $\mu\text{g/day}$ NPIP and 10.4 ± 13.2 (range ND–45.9) mg/day nitrite are observed (Tricker *et al.* 1991c).

Concurrent *Schistosomiasis* infection by *S. haematobium* and *S. mansoni* in Egypt is associated with excretion of 2.74 ± 6.13 $\mu\text{g/day}$ NDMA and significantly increased excretion of 0.26 ± 0.35 $\mu\text{g/day}$ NPYR ($P < 0.02$), 0.21 ± 0.43 $\mu\text{g/day}$ NPIP ($P < 0.05$) and 5.18 ± 9.11 mg/day nitrite ($P < 0.02$) compared with subjects without infection (Tricker *et al.* 1989b). Compared with *S. mansoni* infection, *S. haematobium* infection significantly increases excretion of NDMA (19.2 ± 21.0 vs 2.9 ± 2.9 $\mu\text{g/day}$), NPYR (1.3 ± 1.9 vs 0.9 ± 0.9 $\mu\text{g/day}$) and NPIP (1.6 ± 2.3 vs 0.4 ± 0.3 $\mu\text{g/day}$) (Mostaffa *et al.* 1994). Nitrate excretion is also significantly higher after infection by either *S. mansoni* (249 ± 126 mg/day; $P < 0.001$) or *S. haematobium* (174 ± 176 mg/day; $P < 0.005$) compared with subjects without infection (139 ± 82 mg/day), while nitrite excretion shows a reverse trend with higher levels of excretion after infection by *S. haematobium* compared with *S. mansoni* (7.9 ± 24 vs 3.2 ± 13 mg/day). Excretion of VNA and nitrate persists in subjects with confirmed *Schistosomiasis* infection and diagnosed bilharzial bladder cancer (Tricker *et al.* 1989b).

In comparison with normal bladder urine, rectal urine from ureterosigmoidostomy patients contains significant levels of 0.37 ± 0.35 $\mu\text{g/l}$ NDMA ($P < 0.0001$), 0.20 ± 0.27 $\mu\text{g/l}$ NPYR ($P < 0.001$) and 0.09 ± 0.17 $\mu\text{g/l}$ NPIP ($P < 0.01$) (Tricker *et al.* 1989c). As in the case of NVNA excretion, daily treatment with 2 g ascorbic acid (1 g in children) does not significantly reduce VNA excretion (Kälble *et al.* 1991). Alternative forms of urinary diversion

via an ileal reservoir (Tricker *et al.* 1989c; Gröschel *et al.* 1992) or a Gersuny bladder (Tricker *et al.* 1989c), in which direct contact of urine with faeces is avoided, are not effective in reducing VNA excretion caused by recurrent bacterial infection.

Biomonitoring of urinary N-nitrosamines from other sources

Tobacco and tobacco smoke

The major metabolic pathway of NNK in cultured human tissues involves carbonyl reduction to enantiomeric NNAL (Hecht, 1994) followed by O-glucuronidation to diastereomeric conjugates of [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]- β -D-glucosiduronic acid (NNAL-Gluc) (Murphy *et al.* 1994). Both NNAL and NNAL-Gluc are present in smokers' urine (Table 3) and account for 39–101% of the estimated uptake of NNK (Carmella *et al.* 1993). NNAL-Gluc is assumed to be a urinary detoxification product of NNK metabolism (Hecht, 1994) and total excretion of NNAL plus NNAL-Gluc is considerably influenced by dietary factors (Hecht *et al.* 1995). Intraindividual differences in the NNAL-Gluc:NNAL excretion ratio are generally small ($< 10\%$), whereas 16-fold interindividual differences occur in which two phenotypes of this ratio are apparent (Carmella *et al.* 1995). Strong correlations are observed between urinary NNAL, NNAL-Gluc, and NNAL plus NNAL-Gluc with cotinine excretion ($n = 61$; $R = 0.58$; $P < 0.0001$), but not with the number of cigarettes smoked per day ($R = 0.05$) (Carmella *et al.* 1995). Both NNK metabolites also occur in urine of pipe smokers (B. Pfundstein, personal communication).

Considerably higher urinary excretion of 81 ± 30 (range 43.9–129.7) $\mu\text{g/l}$ NNAL and 341 ± 195 (range 138.9–579.2) $\mu\text{g/l}$ NNAL-Gluc occurs in Sudanese snuff-dippers using toombak tobacco (Murphy *et al.* 1994). Total NNAL plus NNAL-Gluc excretion also correlates ($R = 0.94$; $P < 0.05$) with urinary cotinine. Since toombak tobacco contains both preformed NNK and, to a less extent, NNAL (Prokopczyk *et al.* 1995), excretion of NNAL plus NNAL-Gluc is not a specific biomarker for NNK uptake in toombak users. NNAL and NNAL-Gluc excretion have also been reported in American snuff-dippers and tobacco chewers; however, unlike the situation in smokers, there was no indication of two phenotypes of the NNAL-Gluc:NNAL excretion ratio (Kresty *et al.* 1996). Nasal snuff use also results in urinary excretion of NNAL plus NNAL-Gluc (B. Pfundstein, personal

Table 3. Daily urinary excretion of 4-(methylnitrosamino)-3-(1-pyridyl)-1-butanone (NNK) metabolites in urine of healthy nonsmokers and smokers

Smoking status	n	Cotinine (mg/day)	Urinary excretion of NNK metabolites ($\mu\text{g/day}$)			Reference
			NNAL ^a	NNAL-Gluc ^b	NNAL-Gluc/NNAL ratio ^c	
Nonsmokers	7	—	ND	ND	—	Carmella <i>et al.</i> 1993
Smokers (23.3 \pm 6.8; range 12–36 cigarettes/day)	11	7.73 \pm 5.47 (2.28–20.0)	0.52 \pm 0.26 (0.23–1.0)	3.47 \pm 1.77 (0.57–6.5)	0.5–10.0	
Smokers (13.9 \pm 3.5; range 8–17 cigarettes/day)	9	7.31 \pm 1.72 (4.49–10.1)	0.16 \pm 0.10 (0.04–0.29)	1.07 \pm 0.67 (0.20–1.77)	2.4–4.6	Carmella <i>et al.</i> 1995
Smokers (14.8 \pm 3.4 cigarettes/day)	33	5.83 \pm 2.50	0.15 \pm 0.10	1.00 \pm 0.55	—	Hecht <i>et al.</i> 1995
Smokers (20.9 \pm 4.6; range 8–30 cigarettes/day)	19	4.72 \pm 2.32 (0.59–8.72)	0.31 \pm 0.24 (0.05–0.94)	0.63 \pm 0.36 (0.15–3.10)	0.5 \pm 4.9	McGee <i>et al.</i> 1996

Abbreviations: NNAL, 4-(methylnitrosamino)-3-(1-pyridyl)-1-butanol; NNAL-Gluc, [4-(methylnitrosamino)-3-(1-pyridyl)-1-but-1-yl]- β -D-glucosiduronic acid.

^aTo convert to nmol/day multiply by 4.78.

^bTo convert to nmol/day multiply by 2.59.

^cCalculated on a molar basis.

communication). These results indicate that NNK is metabolized and excreted in the urine of smokers and tobacco users as NNAL and NNAL-Gluc, regardless of the route of uptake.

In an experimental study, excretion of 33.9 ± 20.0 ng/day NNAL plus NNAL-Gluc was observed after 90 min exposure to sidestream cigarette smoke containing 75–263 ng/m³ NNK generated by machine-smoked reference cigarettes (Hecht *et al.* 1993). Although the experimental conditions in which sidestream cigarette smoke was used as a surrogate for ETS were claimed to be comparable to those in a heavily smoke-polluted bar, the same authors (Brunnemann *et al.* 1992) and others (Klus *et al.* 1992; Tricker *et al.* 1994) report 10- to 20-fold lower concentrations of NNK in ETS. Real-life exposure of nonsmokers to ETS would, therefore, not be expected to result in significant amounts of NNAL and/or NNAL-Gluc excretion.

Excretion of 44–163 ng/day iso-NNAC has been reported in four of 19 smokers and not in non-smokers (Tricker *et al.* 1993). The very low levels of iso-NNAC in mainstream cigarette smoke (< 5 ng/cigarette) (Djordjevic *et al.* 1991; Tricker *et al.* 1993) explain why only low levels of iso-NNAC are occasionally found in urine from some smokers.

Cosmetic products

In an experimental study using a single adult male volunteer, application of a facial cosmetic foundation to the back (12.7 g containing 980 μg NDELA) and removal 7.75 h later resulted in a total cumulative urinary excretion of at least 17.3 μg NDELA over a 21.5 h period (one urine fraction was lost for analysis) (Edwards *et al.* 1979). Although this study indicates that dermal absorption and excretion of NDELA occurs in man, normal use of cosmetic products is very unlikely to result in sufficient exposure NDELA to allow its detection in urine (European Chemical Industry Ecology and Toxicology Center, 1990).

Pharmaceutical products

Several hundred pharmaceutical drugs contain secondary and tertiary amino groups potentially susceptible to endogenous nitrosation to yield NDMA and NDEA, and to a lesser extent, NPYR, NPIP, NMOR and *N*-nitrosohexamethylenimine (Lijinsky, 1990). Standardized *in vitro* nitrosation assays performed under simulated gastric conditions (Coulston and Dunne, 1980; Lijinsky, 1990) indicate that several pharmaceutical drugs may react to produce *N*-nitrosamines *in vivo*; however, only in a few cases has confirmation been obtained in man.

Piperazine is readily nitrosated *in vitro* at pH 0.5–5.5 to yield MNPz, and to a lesser extent, *N,N*-dinitrosopiperazine (DNPz) (Mirvish, 1975; Tricker *et al.* 1991d). Extensive metabolism of MNPz occurs in the rat resulting in the excretion of *N*-nitroso-hydroxypyrrolidine (NHPYR) and NDELA (Tricker *et al.* 1991e). Consequently, patients treated with 2 g/day piperazine (contaminated with 3.6 µg MNPz/g piperazine) have been shown to have a mean post-administration excretion of 27.0 ± 26.7 (range 0.6–96.0) µg/day MNPz, 0.73 ± 0.92 (range ND–2.8) µg/day DNPz, 1.74 ± 1.72 (range ND–5.7) µg/day NHPYR, and < 0.3 µg/day NDELA (Tricker *et al.* 1991d). In a follow-up experimental study, dietary supplementation with 250 mg/day nitrate increased the mean MNPz excretion from 36.9 ± 34.1 (range 92–80.1) to 84.1 ± 57.7 (range 63.0–122.7) µg/day in five human volunteers receiving a therapeutic dose of 1.9 g piperazine (contaminated with 4.2 µg MNPz/g piperazine) (Kumar *et al.* 1992). Other experimental studies have shown that administration of 480 mg piperazine in syrup to fasting volunteers results in gastric juice concentrations of 140–230 µg/l MNPz after 30 min, with detectable levels still present at 150 min (Bellander *et al.* 1985). Although partial inhibition of endogenous nitrosation was achieved by co-administration of ascorbic acid, no correlation was found between gastric pH and MNPz formation. Urinary *N*-nitroso compounds derived from the endogenous nitrosation of piperazine probably only represent about 10% of the total level of endogenous formation (Bellander, 1990).

Experimental studies show that the analgesic aminopyrene is readily nitrosated *in vivo* (Spiegelhalter and Preussmann, 1985). Following administration of 500 mg aminopyrene, 40 g ethanol as beer and 250 mg nitrate in radish juice, 8 h urinary excretion of 0.5–10 µg NDMA was observed. The total endogenous formation was estimated to be 25–1,800 µg NDMA based on previous experimental studies on NDMA excretion in man (Spiegelhalter *et al.* 1982) and was only apparent after ethanol consumption to inhibit hepatic metabolism. Similarly, administration of the analgesic metamizol (500 mg) results in excretion of 370 µg/day *N*-nitrosomethyl-aminopyrene, the formation of which is not inhibited by co-administration of ascorbic acid (Ziebarth, 1994). Administration of the antibiotic oxytetracycline (500 mg every 8 h) to patients with bacterial infection of the urinary bladder has also been reported to result in excretion of < 0.16 µg/l NDMA (Webb *et al.* 1983); however, this observation requires confirmation.

Occupational exposure

Machine workers handling NDELA contaminated products can be exposed to NDELA by direct contact or by inhalation of oil mists during most machine shop operations. Excretion of NDELA in exposed workers increases progressively over the course of the working week and correlates with the level of NDELA contamination present in the cutting fluids currently being used (Spiegelhalter *et al.* 1984). In Germany, reductions in NDELA contamination of commercial synthetic cutting fluids are reflected in the observed fall in urinary excretion of NDELA from maximum levels of 103 µg/l urine in 1984 (Spiegelhalter *et al.* 1984) to 0.6 ± 1.6 (range ND–15.0) µg/l in 1992 (Bolm-Audorff *et al.* 1992). At the time of urine collection in 1992, cutting fluids present in the studied workshops contained a mean of 20.6 ± 5.0 (range 2–135) mg/l NDELA resulting in ambient air concentrations of 214 ± 391 (range < 50 –1,000) ng/m³ (Fuchs *et al.* 1995).

Occupational exposure of pharmaceutical production workers to piperazine dust results in endogenous formation and excretion of 0.3–4.7 µg/day MNPz (Bellander *et al.* 1988). MNPz excretion correlates significantly with both breathing zone exposure to piperazine dust (0.06 – 1.7 mg/m³; $R = 0.78$; $P = 0.01$) and urinary piperazine excretion (0.1 – 4.7 mg/day; $R = 0.86$; $P = 0.003$); but not with salivary nitrate and nitrite, or urinary nitrate excretion.

Assessment of endogenous nitrosation by the *N*-nitrosoproline test

The *N*-nitrosoproline test in humans involves sequential administration of nitrate and L-proline to volunteers and determination of endogenous formation of NPRO which is rapidly and quantitatively excreted in urine over the next 24 h (Ohshima and Bartsch, 1981). NPRO excretion is proportional to the proline dose, increases exponentially at a nitrate dose above 186 mg, and can be partially blocked by co-administration of high doses (> 1 g) of ascorbic acid. Several modifications to the original administration protocol (Ohshima and Bartsch, 1981) have been developed for use as an index for assessing the potential for endogenous nitrosation in man (Bartsch *et al.* 1990). The test is considered safe for use in man, since NPRO is noncarcinogenic (Garcia and Lijinsky, 1973; Nixon *et al.* 1975) and quantitatively excreted in rats (Chu and Magee, 1981; Ohshima *et al.* 1982).

The NPRO test in its various forms is not without some inherent weaknesses, in particular the relatively narrow pH range of chemical acid-catalysed

nitrosation of proline (Shuker and Forman, 1991), the nitrosation of which proceeds maximally at pH 2.5 (Mirvish *et al.* 1973), a somewhat lower value than for most amines which show maximal rates at pH 3.0–3.4 (Mirvish, 1975). Other factors which may vary between individuals and affect endogenous NPRO formation include salivary nitrate recirculation (Spiegelhalter *et al.* 1976), nitrate reduction to nitrite by the oral microflora (Shapiro *et al.* 1991) and the availability of gastric nitrosation catalysts such as thiocyanate (Ruddell *et al.* 1977). Regardless of these limitations, the NPRO test has been extensively used to evaluate the potential for increased endogenous nitrosation in the gastric cancer hypothesis, population studies, and in smokers compared with nonsmokers.

The gastric cancer hypothesis

It has been hypothesized that gastric achlorhydria (pH > 4) present in some precancerous conditions such as pernicious anaemia, chronic atrophic gastritis (CAG) and gastric operations (Billroth I and Billroth II partial gastrectomies, truncal vagotomy and proximal-gastral vagotomy), could lead to proliferation of nitrate-reducing bacteria and a subsequent increase in endogenous nitrosation (Correa *et al.* 1975). This hypothesis is supported by positive correlations between fasting gastric juice pH and nitrite levels (Tannenbaum *et al.* 1979; Schlag *et al.* 1980; Kyrtopoulos *et al.* 1985a; Hall *et al.* 1987a; Carbone *et al.* 1988; Knight *et al.* 1991; Pignatelli *et al.* 1987, 1994) and total viable bacterial counts (Kyrtopoulos *et al.* 1985a; Crespi *et al.* 1987; Hall *et al.* 1987; Pignatelli *et al.* 1987; Carbone *et al.* 1988; O'Donnell *et al.* 1988), and the significantly reduced ($P < 0.005$) levels of gastric ascorbic acid in achlorhydric subjects with precancerous gastric pathology (O'Connor *et al.* 1989). Cellular atrophy, loss of gastric glands and their acid-secreting parietal cells and their gradual replacement by intestinal-type glands (intestinal metaplasia) in CAG begins as a multifocal lesion which rapidly spreads to cover much of the gastric mucosa, thus resulting in an increase in gastric pH (to pH 3.0–6.0, depending on the severity of the lesions). Nitrosation within the achlorhydric regions probably results through a non-acid bacterially mediated pathway in which bacterial reduction of nitrate increases the availability of nitrite which can migrate to adjacent areas with normally functioning mucosa where acid-catalysed nitrosation occurs (Leach *et al.* 1987). Alternatively, immunostimulation of the gastric epithelium in response to chronic *Helicobacter pylori* infection may increase

endogenous gastric formation of NO (Shapiro and Hotchkiss, 1996).

Contrary to the gastric cancer hypothesis, the NPRO test has yielded consistent results showing that achlorhydric patient groups have a reduced potential for endogenous NPRO formation when compared with control groups with normal gastric pathology (Table 4). In subjects with severe CAG, intestinal metaplasia or dysplasia, NPRO excretion was significantly reduced ($P < 0.05$) compared with that in subjects with less advanced lesions (Crespi *et al.* 1987). Only in one study was a significant increase ($P < 0.03$) in NPRO excretion observed in achlorhydric patients after administration of proline without additional nitrate (Houghton *et al.* 1989); however, this observation was not confirmed in another study (Xu *et al.* 1993). In subjects monitored for 24 h intragastric pH, NPRO formation was significantly reduced ($P < 0.01$) in achlorhydric subjects (pH > 4.0 > 50% of 24 h) compared with subjects with acidic gastric conditions (pH < 4.0); NPRO excretion was negatively associated with the mean intragastric pH (Hall *et al.* 1987b).

Population studies

Similar to studies on achlorhydric patient collectives (Table 4), studies on populations at risk of gastric cancer have also produced inconsistent and contradictory results (Table 5). In all population studies the NPRO test has been performed without additional nitrate supplementation (Table 5; test condition 1). In some studies the inhibition of endogenous NPRO formation was also studied by co-administration of ascorbic acid (Table 5; test condition 2).

In Japanese subjects from low- and high-prevalence areas for gastric cancer, basal NPRO excretion was not different in undosed subjects; however, after proline administration it increased significantly ($P < 0.001$) only in subjects from the high-prevalence area (Kamiyama *et al.* 1987). Intake of ascorbic acid inhibited the increase in NPRO excretion only in the high-prevalence population. Since nitrate excretion in the absence of proline administration was significantly higher ($P < 0.07$) in the low-prevalence population, the potential for intragastric nitrosation appears to be considerably higher in subjects living in the high-prevalence area for gastric cancer. In Poland no difference was found between basal NPRO and nitrate excretion in low- and high-prevalence populations for gastric cancer; however, NPRO excretion after proline administration increased more in the high-prevalence population (Zatonski *et al.* 1989). In Costa Rica endogenous

Table 4. Evaluation of the gastric cancer hypothesis using the *N*-nitrosoproline test

Patient group	n	Test condition (mg) ^a		Urinary excretion of <i>N</i> -nitrosoproline (µg/day)				<i>p</i> ^b	Reference
		Nitrate	L-Proline	Range	Mean ± SD	Median	95% CI		
Control	9	380	500	—	18.0 ± 7.2	—	—	—	Hall <i>et al.</i> , 1987b
Pernicious anaemia	10	380	500	—	1.1 ± 0.8	—	—	< 0.01	
Polygastrectomy	10	380	500	—	3.2 ± 2.3	—	—	NS	
Control (pH < 4.0)	10	380	500	—	17.9 ± 6.6	—	—	—	
Achlorhydria (pH > 4.0)	13	380	500	—	0.9 ± 0.6	—	—	< 0.01	
Control	18	300	500	—	—	3.7	1.8–7.9	—	Knight <i>et al.</i> , 1991
Superficial gastritis	8	300	500	—	—	1.6	0.5–5.0	NS	
CAG	21	300	500	—	—	3.4	1.7–6.8	NS	
Gastric cancer	6	300	500	—	—	3.0	0.1–10.8	NS	
Control (pH < 4.0)	22	300	500	—	—	4.1	2.1–8.0	—	
Achlorhydria (pH > 4.0)	31	300	500	—	—	2.5	1.5–4.3	NS	
Control/superficial gastritis (pH < 4.0)	12	260	500	—	—	15.8	10.3–26.0	—	Crespi <i>et al.</i> , 1987
Mild CAG (pH < 4.0)	12	260	500	—	—	17.3	9.5–27.9	NS	
Severe CAG (pH < 4.0)	11	260	500	—	—	9.0	4.2–21.5	NS	
Control/superficial gastritis (pH < 8.0)	15	260	500	—	—	14.4	6.4–17.9	—	
Mild CAG (pH < 8.0)	17	260	500	—	—	12.1	4.5–20.4	NS	
Severe CAG (pH < 8.0)	18	260	500	—	—	6.3	2.9–9.3	< 0.05	
Control	16	218	500	1.7–11.8	—	2.9	—	—	Houghton <i>et al.</i> , 1989
Vagotomy	14	218	500	0.6–22.3	—	3.7	—	NS	
Partial gastrectomy	12	218	500	0.2–14.5	—	2.1	—	NS	
Pernicious anaemia	9	218	500	1.3–8.1	—	3.8	—	NS	
Control	22	200	500	ND–33.5	8.5 ± 7.8	—	—	—	Adam <i>et al.</i> , 1989
Bilroth I	5	200	500	ND	—	—	—	< 0.05	
Bilroth II	26	200	500	ND–9.3	1.2 ± 2.7	—	—	< 0.05	
Proximal vagotomy	6	200	500	ND–22.9	10.5 ± 8.1	—	—	NS	
Control	20	—	500	ND–4.86	—	0.93	—	—	Houghton <i>et al.</i> , 1989
Vagotomy	9	—	500	1.1–9.1	—	1.75	—	0.03	
Partial vagotomy	15	—	500	0.29–9.18	—	2.62	—	0.003	
Pernicious anaemia	11	—	500	ND–9.15	—	1.44	—	NS	
Control	29	—	300	—	9.27 ± 7.38	—	—	—	Xu <i>et al.</i> , 1993
Intestinal metaplasia	31	—	300	—	8.02 ± 5.24	—	—	NS	
Gastric cancer or dysplasia	26	—	300	—	7.77 ± 6.19	—	—	NS	

Abbreviations: NPRO, *N*-nitrosoproline; CAG, chronic atrophic gastritis; ND, not detected.^aRefer to test for NPRO test conditions.^bAll tests of significance compare control group with different gastric pathology groups.

Table 5. Evaluation of endogenous nitrosation potential in epidemiological studies using the *N*-nitrosoproline test

Population and comment		Test conditions (mg)		Urinary excretion of nitrate (mg) and <i>N</i> -nitrosoproline (μg/day)						Reference	
		<i>n</i>	<i>L</i> -Proline	Ascorbic acid	Background excretion		Test condition 1 ^a		Test condition 2 ^b		
					Nitrate	NPRO	Nitrate	NPRO	Nitrate		NPRO
Costa Rica (12 h excretion; children)											
Low gastric cancer prevalence	25	1 × 500	1 × 200	–	–	12.4 [9.9–25.4]	0.54 [0.36–0.86]	14.3 [11.2–25.4]	0.28 [0.20–0.36]	Sierra <i>et al.</i> 1993	
High gastric cancer prevalence	26	1 × 500	1 × 200	–	–	14.3 [9.3–27.3]	0.84* [0.51–1.67]	18.0 [12.4–32.9]	0.66* [0.31–0.98]		
Italy (12 h excretion; adults)											
Low gastric cancer prevalence	40	1 × 500	–	33.7 [27.0–42.0]	0.58 [0.4–0.84]	34.4 [28.8–41.0]	1.44 [1.1–1.87]	–	–	Knight <i>et al.</i> 1992	
High gastric cancer prevalence	40	1 × 500	–	37.1 [29.7–46.3]	0.94* [0.7–1.24]	36.0 [29.7–43.6]	1.59 [1.2–2.08]	–	–		
Poland (24 h excretion; adults)											
Low gastric cancer prevalence	47	3 × 100	3 × 100	90 [7–344]	2.0 [0.4–19.3]	76 [9–239]	2.4 [0.1–18.9]	70 [22–377]	1.8 [0.1–8.9]	Zatonski <i>et al.</i> 1989	
High gastric cancer prevalence	50	3 × 100	3 × 100	96 [36–475]	1.8 [ND–23.4]	118** [29–354]	2.8 [0.1–10.9]	104* [16–428]	1.2 [1.7–2.9]		
Japan (24 h excretion; adults)											
Low gastric cancer prevalence	52	3 × 100	3 × 100	145 [118–170]	6.1 [4.1–8.1]	117 [136–219]	7.1 [6.6–9.6]	88 [61–115]	4.9 [3.3–6.5]	Kamiyama <i>et al.</i> 1987	
High gastric cancer prevalence	52	3 × 100	3 × 100	95*** [73–117]	3.8 [2.6–5.0]	116 [83–150]	12.6*** [8.5–16.7]	130 [107–160]	3.2* [2.4–4.0]		
China (24 h excretion; adults)											
Low oesophageal cancer prevalence	40	3 × 100	3 × 100	48 [37–61]	1.7 [0.9–2.9]	57 [38–77]	4.4 [3.3–6.4]	–	–	Lu <i>et al.</i> 1986	
High oesophageal cancer prevalence	40	3 × 100	3 × 100	94*** [69–119]	5.7*** [4.1–7.7]	85*** [66–114]	8.3*** [6.4–12.3]	89 [58–115]	2.4 [1.7–2.9]		
Low oesophageal cancer prevalence	44	3 × 100	3 × 100	–	2.0 ± 2.6	–	3.4 ± 4.5	–	3.1 ± 4.4	Lu <i>et al.</i> 1987	

Table 5 (continued)

Population and comment	n	Test conditions (mg)		Urinary excretion of nitrate (mg) and N-nitrosoproline (μg/day)						Reference
		L-Proline	Ascorbic acid	Background excretion		Test condition 1 ^a		Test condition 2 ^b		
				Nitrate	NPRO	Nitrate	NPRO	Nitrate	NPRO	
High oesophageal cancer prevalence	44	3 × 100	3 × 100	-	5.7 ± 8.2	-	10.2 ± 20.7	-	3.6 ± 5.9	
	47	3 × 100	3 × 100	-	5.9 ± 9.2	-	12.2 ± 22.7	-	4.1 ± 4.7	
	51	3 × 100	3 × 100	-	4.2 ± 6.3	-	7.0 ± 11.8	-	2.8 ± 3.1	
	57	3 × 100	3 × 100	-	6.6 ± 11.2	-	11.3 ± 24.9	-	3.5 ± 6.8	
	47	3 × 100	3 × 100	-	4.4 ± 6.2	-	7.3 ± 8.3	-	4.6 ± 4.6	
China										
(12 h excretion; adults)										
Low nasopharyngeal cancer prevalence	40	1 × 500	1 × 300	38 ± 6	3.3 ± 0.8	24 ± 5	3.2 ± 1.2	28 ± 4	2.4 ± 0.8	Zeng <i>et al.</i> , 1993
High nasopharyngeal cancer prevalence	37	1 × 500	1 × 300	54 ± 9 ^c	3.9 ± 0.9	125 ± 19 ^{***}	7.6 ± 2.9	70 ± 15 ^{***}	3.4 ± 0.8	
26 Counties study	1035	1 × 500	1 × 200	-	-	260 ± 130 (90-490)	12.4 ± 10.0 (2.9-45.3)	-	6.35 ± 7.75 (0.9-39.6)	Chen <i>et al.</i> , 1987
69 Counties study	4000	1 × 500	1 × 200	-	-	170 ± 70 (50-360)	13.8 ± 11.9 (1.1-69.0)	-	5.08 ± 3.86 (0.6-19.4)	Wu <i>et al.</i> , 1993
Thailand										
(12 h excretion; adults)										
Low cholangiocarcinoma cancer prevalence	18	1 × 500	1 × 200	-	-	57.7 ± 55.8	9.0 ± 14.8	35.3 ± 27.9	1.3 ± 2.0	Haswell-Elkins <i>et al.</i> , 1994
Intermediate cholangiocarcinoma prevalence	20	1 × 500	1 × 200	-	-	63.9 ± 47.7	6.3 ± 8.3	58.3 ± 37.2	5.3 ± 10.3	
High cholangiocarcinoma cancer prevalence	20	1 × 500	1 × 200	-	-	53.3 ± 49.0	4.2 ± 3.5	4.6 ± 36.0	11.5 ± 20.2	
	21	1 × 500	1 × 200	-	-	70.2 ± 35.3	2.9 ± 2.5	98.6 ± 71.9	1.8 ± 1.4	
	20	1 × 500	1 × 200	-	-	52.7 ± 54.6	14.2 ± 19.4	62.6 ± 42.8	3.0 ± 2.1	
Seronegative for <i>O. viverrini</i> antibody	18	1 × 500	1 × 200	-	-	62.6 ± 56.4	3.5 ± 3.2	97.3 ± 71.3	2.5 ± 1.6	
Seropositive for <i>O. viverrini</i> antibody	23	1 × 500	1 × 200	-	-	60.8 ± 29.8	12.3 ± 18.7 ^{a,c}	68.2 ± 50.2	2.4 ± 2.0	

Abbreviations: NPRO, N-Nitrosoproline; ND, not detected.

Data reported as either arithmetic means ± SD (range) or geometric means [95% confidence limits]

All tests of significance compare areas of low- and high-prevalence for specific cancers unless otherwise stated: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.^aTest condition 1: administration of L-proline^bTest condition 2: administration of L-proline plus ascorbic acid.^cCompares seronegative against seropositive subjects.

Table 6. Evaluation of endogenous nitrosation potential in nonsmokers and smokers by using the *N*-nitrosoproline test

Experimental conditions and administration of test compounds (mg per administration)				NPRO excretion (µg/day)								
Controlled diet	Nitrate	L-Proline	Ascorbic acid	Nonsmokers			Smokers			P ^a	Reference	
				n	Mean ± SD	Range	n	Mean ± SD	Range			
Yes	-	-	-	13	3.55 ± 2.13	0.9-9.3	13	5.90 ± 4.36	1.5-14.6	< 0.05	Hoffmann and Brunnemann, 1983	
Yes	-	300	-	14	3.61 ± 1.63	1.4-5.9	14	11.7 ± 15.5	0.7-49.3	< 0.05		
Yes	-	300	250	13	4.68 ± 2.51	ND-9.2	13	4.56 ± 3.73	ND-12.5	NS		
Yes	-	-	250	9	3.97 ± 3.48	0.6-12.4	8	5.96 ± 5.29	1.0-15.2	NS		
Yes	-	-	-	12	1.7 ± 0.4	ND-4.3	16	2.1 ± 0.5	ND-6.7	NS	Ladd <i>et al.</i> , 1984	
Yes	-	500	-	9	1.9 ± 0.7	ND-5.8	10	2.4 ± 0.6	ND-4.6	NS		
Yes	325	500	-	12	16.8 ± 3.6	6.4-47.6	16	42.6 ± 7.8	7.4-130.6	< 0.05		
No	-	-	-	4	0.32 ± 0.14	-	6	0.81 ± 0.51	-	NS	Nair <i>et al.</i> , 1986	
No	-	3 × 100	-	4	0.55 ± 0.12	-	6	2.84 ± 1.27	-	NS		
No	-	500	-	10	1.6 ± 0.6	ND-5.1	10	6.1 ± 2.1	ND-19.6	< 0.05	Scherer and Adikofer, 1986	

Abbreviations: NPRO, *N*-nitrosoproline; ND, not detected.^aSignificant difference between nonsmokers and smokers.

NPRO formation was significantly higher in children living in a high-prevalence area for gastric cancer than in those living in a low-prevalence area (Sierra *et al.*, 1993). NPRO excretion on the day of proline administration correlated significantly ($P < 0.001$) with the urinary nitrate level, but not when ascorbic acid was ingested. In Italy basal NPRO and nitrate excretion was higher in the high-prevalence population; however, contrary to the above studies, a reduced potential for endogenous NPRO formation was found after administration of proline (Knight *et al.*, 1992). In Chinese subjects, no clear relationship was found between NPRO excretion and gastric mortality (Chen *et al.*, 1987).

Oesophageal cancer mortality rates in China are positively and significantly associated with NPRO excretion after both administration of proline ($P < 0.01$) and proline plus ascorbic acid ($P < 0.01$) (Wu *et al.*, 1993). Significantly higher NPRO ($P = 0.001$) and nitrate ($P = 0.001$) excretion was observed in a high-prevalence area, both prior to and after administration of proline, compared with a low-prevalence area; however, after administration of proline, the increase in NPRO excretion was more significant in the low-prevalence ($P = 0.001$) compared with the high-prevalence ($P = 0.02$) population (Lu *et al.*, 1986). Contrary to this, the same authors have also reported that the increase in NPRO excretion after proline administration was significantly higher in several high-prevalence populations compared with a low-prevalence population (Lu *et al.*, 1987).

In low- and high-prevalence areas for nasopharyngeal cancer in China, no significant difference was observed in basal excretion of NPRO; however, NPRO excretion after administration of proline was significantly higher ($P = 0.09$) in the high-prevalence population (Zeng *et al.*, 1993). After correlation for urinary creatinine, the difference was not significant.

In contrast to the reported elevated basal excretion of NPRO and nitrate in subjects with *O. viverrini* infection, a risk factor for cholangiocarcinoma in Thailand (Sriantjara *et al.*, 1987), no clear increase in endogenous NPRO formation was observed after administration of proline and proline plus ascorbic acid to subjects living in five areas of contrasting incidence of cholangiocarcinoma (Srivatanakul *et al.*, 1991). When subjects from high-prevalence areas were classified as either seronegative or seropositive for *O. viverrini* antibody and results corrected for urinary creatinine, no significant differences were observed.

Smokers and nonsmokers

Four studies have used the NPRO test to evaluate specifically whether smokers have an increased potential for endogenous nitrosation compared with nonsmokers (Table 6). Under experimentally controlled dietary conditions, single bolus administration of proline (300 mg) results in significantly higher ($P < 0.05$) NPRO excretion in smokers compared with nonsmokers, formation of which was totally inhibited by coadministration of ascorbic acid (250 mg) (Hoffmann and Brunnemann, 1983). Under normal dietary conditions, administration of proline after each meal (100 mg) resulted in slightly higher, but not significantly increased, excretion of NPRO in smokers (Nair *et al.*, 1986). Bolus administration of proline (500 mg) under normal dietary conditions, however, resulted in a significant increase ($P < 0.05$) in NPRO excretion by smokers (Scherer and Adlkofer, 1986), but not under controlled dietary conditions (Ladd *et al.*, 1984). Only when nitrate (325 mg/kg) was administered, together with proline, under controlled dietary conditions was the increase in NPRO excretion significantly higher ($P < 0.05$) in smokers (Ladd *et al.*, 1984). The contradictory results obtained using different test and experimental protocols provide no clear indication that smoking is associated with an increased potential for endogenous nitrosation.

Other studies in which the influence of smoking was considered also produce contradictory results as to whether smoking increases the potential for endogenous nitrosation. In an experimental study in which subjects consumed a nutritionally normal diet but controlled for intake of nitrate, no significant effect to smoking on urinary NPRO excretion was observed after administration of proline (500 mg) and ingestion of a high-nitrate salad (168 mg NO_3^-), with and without additional vitamin C (325 mg) supplementation (Knight and Forman, 1987). No effect of smoking was found in a Chinese population at risk for oesophageal cancer (Wu *et al.*, 1993). Smoking appeared to increase the potential for endogenous NPRO formation in patients with achlorhydria (Crespi *et al.*, 1987; Knight *et al.*, 1991); however, in populations living in high-prevalence areas for gastric cancer identical test conditions resulted in increased NPRO excretion in Japanese smokers (Kamiyama *et al.*, 1987), but not in Polish smokers (Zatonski *et al.*, 1989).

Apparent total N-nitroso compounds

The determination of individual N-nitrosamines in body fluids provides only a partial assessment of the

Table 7. Apparent total *N*-nitroso compounds (ATNC) in gastric juice as a function of pH^a

Patient group	n	pH	ATNC ($\mu\text{mol/l}$)		Reference
			Mean \pm SD	Range	
Patients before and after operations for duodenal ulcers or with chronic atrophic gastritis	11	1.0-1.5	0.68 \pm 0.48	0.10-1.69	Pignatelli <i>et al.</i> , 1987
	25	1.6-2.3	0.46 \pm 0.74	ND-3.60	
	10	2.5-3.3	0.38 \pm 0.68	ND-2.25	
	4	3.6-5.0	1.41 \pm 0.92	0.06-2.13	
	11	5.7-7.0	1.28 \pm 1.71	0.05-6.00	
	12	7.2-8.9	0.46 \pm 0.59	ND-2.00	
Gall bladder	15	1.3-2.0	0.39 \pm 0.77	ND-3.10	Kyrtopoulos <i>et al.</i> , 1991
	7	2.2-3.8	2.05 \pm 2.69	0.10-6.20	
	6	4.6-7.6	0.55 \pm 0.55	ND-1.30	
Normal	30	1.1-3.5	0.41	0.01-2.29	Pignatelli <i>et al.</i> , 1991
Achlorhydric	12	4.5-8.5	0.18	ND-0.50	

Abbreviations: ATNC, apparent total *N*-nitroso compounds; ND, not detected.

^aAll studies performed using the Pignatelli method to determine ATNC (Pignatelli *et al.*, 1987)

extent of *N*-nitrosamine exposure because currently available methods are limited to determining known metabolically stable compounds and/or their metabolites. Group selective determination by chemical denitrosation and detection of released NO provides an estimate of the total concentration of all *N*-nitroso compounds present within a biological matrix, but provides no information about the identities or levels of individual species (Massey, 1988). However, information may be provided for the presence of *N*-nitroso compounds for which analysis procedures have not been established, provided care is taken to avoid both false-positive and false-negative interferences (Pignatelli and Walters, 1996). For this reason and the fact that the majority of the released NO stems from unknown *N*-nitroso compounds, the term 'apparent total *N*-nitroso compounds' (ATNC) is used.

Gastric juice

The analysis of ATNC in gastric juice is subject to some controversy, primarily because different analytical techniques give significantly different results. Using the Walters method in which ATNC are extracted into ethyl acetate prior to determination (Walters *et al.*, 1978a), a positive relationship is found between fasting gastric juice pH and ATNC (Reed *et al.*, 1981a,b; Stockbrugger *et al.*, 1982; Sharma *et al.*, 1984). According to these studies, fasting gastric juice of normal acidity (pH < 4.0) contains 0.1-0.3 $\mu\text{mol/l}$ ATNC while achlorhydria is associated with an increase to about 1.0-1.5 $\mu\text{mol/l}$. These data are in agreement with the gastric cancer hypothesis (Correa *et al.*, 1975); however, the

increase in gastric ATNC in achlorhydric patients was not always observed (Kyrtopoulos *et al.*, 1985a). Using the method of Bavin *et al.* (1982), which does not involve prior extraction, no relationship was observed between gastric juice pH and ATNC (Milton-Thompson *et al.*, 1982; Keighley *et al.*, 1984; Hall *et al.*, 1986). In these studies, samples of gastric juice were obtained frequently following consumption of a meal rather than fasting gastric juice, and the results obtained are approximately 5-fold higher than those using the Walters method.

In a comparison of the methods of Walters and Bavin using 146 fasting gastric juice samples (Dang Vu *et al.*, 1994), a significant positive correlation ($R = 0.626$, $P < 0.01$) was found between gastric juice pH and nitrite concentrations: ATNC determined by the Walters method gave a weak positive correlation ($R = 0.206$, $P < 0.02$) with gastric pH; however, not when determined using a slight modification of the Bavin method (Dang Vu *et al.*, 1983). Only a small fraction of gastric ATNC could be extracted into ethyl acetate (9.1% at pH < 4.0 and 22.1% at pH \geq 4.0) when using the Walters method, which may explain why the Bavin method gives approximately 5-fold higher results. However, the Dang Wu method also measures some compounds that are not *N*-nitroso compounds. Similarly, the Bavin method is also affected by false-positive responses from certain *C*-nitroso compounds and *S*-nitrothiols (Barnard *et al.*, 1982). The method of Walters *et al.* (1978b) is also susceptible to false-positive responses from nitrolic acids and *S*-nitrothiols, in addition to false-negative responses caused by losses of non-extracted ATNC. A photolytic denitro-

sation method for the determination of released NO from gastric juice and urine has also been reported (Janini *et al.*, 1993). Using this method significantly higher ATNC concentrations ($P < 0.01$) and nitrite levels ($P < 0.01$) are reported in subjects with gastric pH > 2.4 (You *et al.*, 1996). Regardless of gastric pH, ATNC concentrations were higher in women than in men (0.16 vs 0.11 $\mu\text{mol/l}$; $P < 0.05$). False-positive responses from inorganic nitrite and nitrate may also make a significant confounding contribution to the release of NO in all the above methods.

Several of the problems inherent to these methods appear to have been partially overcome using the method of Pignatelli *et al.* (1987). Representative data obtained using this method are summarized in Table 7. No significant difference was found in gastric juice ATNC with either extreme acid or basic pH (0.6 $\mu\text{mol/l}$ ATNC at pH ≤ 3.3 ; 0.5 $\mu\text{mol/l}$ ATNC at pH ≥ 7.2), while samples at pH 3.6 – 7.0 showed an average concentration of 1.3 $\mu\text{mol/l}$ ATNC (Pignatelli *et al.*, 1994). These results (both means and ranges of ATNC) are in partial agreement with those reported using the Walters method and provide additional support for the gastric cancer hypothesis. However, achlorhydria was not always associated with increased levels of gastric ATNC (Kyrtopoulos *et al.*, 1991; Pignatelli *et al.*, 1991; Houben *et al.*, 1996).

All five of the above methods (Walters *et al.*, 1978a; Bavin *et al.*, 1982; Dang Wu *et al.*, 1983; Pignatelli *et al.*, 1987; Janini *et al.*, 1993) suffer from the limitation that they do not measure the total level of ATNC as a group. All these methods are relatively slow, require nitrite scavengers, and are unlikely to measure reactive labile compounds. Further modification of the Pignatelli method has produced a method that appears to satisfy these limitations (Xu and Reed, 1993a). Using this method confirms that two separate significantly higher ATNC peaks, one at low pH (range 1.1 – 2.99 ; mean ATNC 1.28 ± 0.15 $\mu\text{mol/l}$) and the other at high pH (range 6.0 – 7.9 ; mean ATNC 3.74 ± 0.59 $\mu\text{mol/l}$) in which nitrite concentrations are strongly related to pH ($R = 0.48$; $P < 0.01$) (Xu and Reed, 1993b). Subsequent studies (Xu and Reed, 1993c) have confirmed the linear relationship between nitrite concentrations and gastric pH ($R = 0.887$; $P < 0.01$) above pH 5 and the presence of two ATNC peaks at low pH (range 1.1 – 2.99 ; mean ATNC 1.45 ± 0.17 $\mu\text{mol/l}$; $P < 0.05$) and at high pH (range 6.0 – 8.4 ; mean ATNC 3.57 ± 0.33 $\mu\text{mol/l}$; $P < 0.01$). The data from more than 1,000 samples of fresh gastric juice have been analysed using this method, confirming

the presence of two ATNC peaks, one at low pH (2 – 3) and the other at high pH (6 – 8), reflecting a significant relationship between clinical conditions associated with increased gastric cancer risk and gastric ATNC levels (Reed *et al.*, 1996). Thus providing further support for the Correa hypothesis (Correa *et al.*, 1975). Representative data using this method are summarized in Table 8.

The formation of ATNC during *in vitro* incubation of human gastric juice with nitrite shows first-order dependence on nitrite and a pH profile with a sharp and continuous increase in rate below pH 3.0 (Kyrtopoulos *et al.*, 1985b). This indicates that gastric ATNC are predominantly formed by nitrosation of weakly basic nitrogenous compounds (Kyrtopoulos, 1989), in agreement with theoretical studies (Shephard *et al.*, 1987). The total *in vivo* gastric flux of ATNC may be as high as 5 $\mu\text{mol/day}$ (Kyrtopoulos *et al.*, 1991), corresponding to about 720 $\mu\text{g/day}$ NPRO. Enterohepatic recycling of ATNC probably accounts for the observed levels in bile (Caygill *et al.*, 1991).

Faeces

Using the Walters method, large differences are found in faecal ATNC excretion which range between 0.91 – 13.41 and 2.27 – 5.68 $\mu\text{mol/kg}$ ATNC in men and women, respectively (Rowland *et al.*, 1991). Total daily excretion ranges between 0.07 – 2.27 and 0.11 – 0.61 $\mu\text{mol/day}$ ATNC in men and women, respectively, with a 13-fold intraindividual variation (0.16 – 2.16 $\mu\text{mol/day}$ ATNC). In an experimental study involving eight volunteers without dietary restriction, the mean excretion for each subject on three different days ranged between 0.45 – 5.33 $\mu\text{mol/day}$ ATNC with a grand mean of 1.56 ± 1.56 $\mu\text{mol/day}$ ATNC. After consumption of a low-nitrate diet (11.0 – 11.6 mg nitrate/day) for 8 continuous days, faecal excretion decreased to 0.28 ± 0.26 (range ND – 0.75) $\mu\text{mol/day}$ ATNC and increased to 1.36 ± 1.39 (range 0.13 – 4.22) $\mu\text{mol/day}$ ATNC after supplementation of the low-nitrate diet with an oral dose of nitrate (300 mg) for 4 continuous days. Only under controlled dietary conditions with oral nitrate supplementation was a positive correlation ($R = 0.70$; $P < 0.1$) found between faecal ATNC and urinary nitrate excretion.

Using the Pignatelli method, faecal excretion was reported to be 10.56 ± 8.60 (range 1.3 – 33.1) $\mu\text{mol/kg}$ ATNC (Pignatelli *et al.*, 1990). Using this method, faecal ATNC concentrations are considerably higher than those reported in gastric juice (Table 7), since

Table 8. Nitrate and apparent total *N*-nitroso compounds (ATNC) in gastric juice as a function of pH^a

Patient group	n	pH	Nitrate ($\mu\text{mol/l}$) Mean \pm SE	ATNC ($\mu\text{mol/l}$) Mean \pm SE	Reference
Normal	54	2.5	4.1 \pm 3.0	1.6 \pm 0.3	Reed <i>et al.</i> 1993
Duodenal ulcer	20	1.9	0.3 \pm 0.1	1.6 \pm 1.2	
Gastric ulcer	8	2.4	2.9 \pm 2.5	2.5 \pm 1.0	
Vagotomy and pyloroplasty	19	3.4	6.4 \pm 3.9	2.2 \pm 0.7	
Chronic atrophic gastritis	11	5.5	24.0 \pm 11.8	2.8 \pm 1.0	
Partial gastrectomy	40	6.5	19.8 \pm 3.5	2.2 \pm 0.4	
Pernicious anaemia	11	7.5	68.6 \pm 11.7	2.8 \pm 0.8	
Gastric cancer	9	4.9	144.5 \pm 78.0	4.7 \pm 2.1	
Normal	44	2.9	3.9 \pm 1.6	1.3 \pm 0.2	Reed <i>et al.</i> 1995
Duodenal ulcer	16	1.9	0.6 \pm 0.6	1.2 \pm 0.5	
Gastric ulcer	10	3.0	9.6 \pm 9.6	1.8 \pm 0.7	
Vagotomy and pyloroplasty	11	4.1	28.4 \pm 15.0	1.7 \pm 0.4	
Chronic atrophic gastritis	14	5.1	29.4 \pm 7.5	3.0 \pm 0.8	
Partial gastrectomy	9	7.5	81.4 \pm 30.1	3.9 \pm 0.7	
Pernicious anaemia	9	8.2	55.0 \pm 5.9	3.3 \pm 1.0	
Gastric cancer	3	7.7	25.0 \pm 9.5	7.1 \pm 0.7	

Abbreviations: ATNC, apparent total *N*-nitroso compounds.^aAll studies performed using the improved Pignatelli method to determine ATNC (Xu and Reed, 1993).

ATNC concentrations are 10-fold higher in the large intestine than in the stomach of rats (Massey *et al.*, 1988), it appears that additional endogenous formation of ATNC may also occur in the large intestine.

Urine

Using the Walters method, bacterial infection of the urinary bladder increases urinary excretion from 0.71 ± 1.13 (range 0.01–2.93) $\mu\text{mol/l}$ ATNC to 1.69 ± 2.86 (range 0.03–10.81) $\mu\text{mol/l}$ ATNC (Ohshima *et al.* 1987). Urinary excretion of 0.13 ± 0.11 (range ND–0.40) $\mu\text{mol/l}$ ATNC in Egyptian subjects was significantly increased ($P < 0.05$) to 0.23 ± 0.18 (range 0.05–0.70) $\mu\text{mol/l}$ ATNC in patients with bacteriuria and 0.20 ± 0.41 (range 0.01–1.86) $\mu\text{mol/l}$ ATNC when accompanied by *S. haematobium* infection (Hicks *et al.* 1982). As discussed previously, the Walters method probably underestimates the actual ATNC concentration in urine. Similarly, inadequate analytical methodology probably accounts for the low level of 0.28 ± 0.14 (range 0.06–0.61) $\mu\text{mol/l}$ ATNC reported for sterile urines in another study (Cox *et al.* 1982).

Using the Pignatelli method, healthy adults have a urinary excretion of 1.00 ± 0.81 (range 0.11–3.20) $\mu\text{mol/l}$ ATNC (Pignatelli *et al.* 1989). Following administration of proline (500 mg), 12 h urine samples contained 0.82 ± 0.65 (range 0.20–2.63) $\mu\text{mol/l}$ ATNC in which total excretion of NVNA

(NSAR, NPRO, NTCA and NMTCa) accounted for 16.3 ± 7.7 (range 4.7–25.9)% of the ATNC response. Median urinary ATNC excretion is significantly lower in healthy subjects (0.31 $\mu\text{mol/day}$) than in patients with Kock (0.63 $\mu\text{mol/day}$; $P = 0.0001$) and hemi-Kock (0.50 $\mu\text{mol/day}$; $P = 0.0001$) urinary diversions (Pignatelli *et al.* 1996).

Using the Janini method, the presence of CAG, intestinal metaplasia and dysplasia has little effect on urinary excretion of ATNC, NPRO, NTCA and NMTCa; however, total ATNC levels were significantly higher ($P < 0.05$) in women than in men (You *et al.* 1996).

Discussion

Prior to the American National Academy of Sciences assessment of human exposure to preformed *N*-nitroso compounds in 1981 (National Academy of Sciences, 1981), only eight *N*-nitrosamines had been identified in foods and beverages compared with the 25 *N*-nitroso compounds now known to occur in the diet (Sen, 1988; Tricker and Preussmann, 1991a; Lillard and Hotchkiss, 1994; Pensabene and Fiddler, 1994; Sen *et al.* 1995). Analytical studies show that about 15% of the ATNC response of various foods can be accounted for by currently identified *N*-nitrosamines (Tricker *et al.* 1985; Massey *et al.* 1991) and that most of the unidentified ATNC response is due to insoluble

protein-bound species (Tricker *et al.* 1985), such as NPRO present in cured meats (Sen *et al.* 1989). As a consequence, both the 1981 and subsequent assessments of a total dietary exposure to 10–100 $\mu\text{g/day}$ NVNA (Hotchkiss, 1989; Tricker and Preussmann, 1991a) may still be conservative. More recent studies in the UK estimate an adult to have a total mean dietary exposure to 0.79 $\mu\text{mol/day}$ ATNC (Ministry of Agriculture, Fisheries and Food, 1992). This ATNC level is equivalent to 114 $\mu\text{g/day}$ NPRO, suggesting that average total daily exposure to N-nitrosamines in the diet is most probably in the region of 80–120 $\mu\text{g/day}$.

Data available prior to 1981 indicated that TSNA in mainstream cigarette smoke contributed 16.2 $\mu\text{g/day}$ to the total smoking-related exposure of 17.0 $\mu\text{g/day}$ N-nitrosamines (Hoffmann *et al.* 1979, 1980). Although TSNA are still quantitatively the major N-nitroso compounds present in cigarette smoke (Tricker *et al.* 1991b), more current data for European and North American filter cigarettes (about 95% of the commercial cigarette market) show significant reductions in TSNA levels (Fischer *et al.* 1990a,b; Tricker *et al.* 1991b). As a result, smoking 20 cigarettes/day is now estimated to result in an exposure of about 3.0 $\mu\text{g/day}$ TSNA and 0.4 $\mu\text{g/day}$ VNA (Tricker *et al.* 1991b). This corresponds to about 0.02 $\mu\text{mol/day}$ N-nitroso compounds, equivalent to only 3.0% of the total daily dietary intake of ATNC.

Occupational exposure to N-nitrosamines was evaluated to be 10–180 $\mu\text{g/day}$ in 1981 (National Academy of Sciences, 1981), and still remains the major source of exposure in most countries without occupational health and safety standards. Based on the latest NIOSH survey (Rey and Fajen, 1996), worker exposure to 5.25 ± 3.41 (range 0.76–16.3) $\mu\text{g/m}^3$ total VNA in breathing zone air samples collected in the rubber industry indicates a mean 8 h exposure to 35.0 ± 22.7 (range 5.1–109.6) $\mu\text{g/day}$ total VNA. This corresponds to 0.41 ± 0.28 (range 0.06–1.32) $\mu\text{mol/day}$ total VNA assuming a respiratory volume of 20 m^3/day . Under poor working conditions in the former USSR, maximum total exposure of 680 $\mu\text{g/day}$ VNA (about 7 $\mu\text{mol/day}$) in some areas of the rubber industry may still occur (Sokoloskaia *et al.* 1993). Under good working conditions, in which occupational health and safety standards are maintained, air monitoring indicates an exposure of <0.1 $\mu\text{g/kg}$ body weight/day (about 0.08 $\mu\text{mol/day}$) VNA (Wolf, 1989). Despite improvement of working conditions in the metal working industry, urinary biomonitoring shows exposure to 20 $\mu\text{g/day}$

(0.15 $\mu\text{mol/day}$) NDELA may still occur (Bohm-Audorf *et al.* 1992). Therefore, occupational exposure is estimated to be about 0.15–0.30 $\mu\text{mol/day}$, which corresponds to about 38% of the total daily dietary intake of ATNC.

Compared with the 1981 exposure estimate of 0.4 $\mu\text{g/day}$ N-nitrosamines caused by the use of cosmetics (National Academy of Sciences, 1981), normal use of cosmetics and personal care products are currently considered to be a negligible source of N-nitrosamine exposure (European Chemical Industry Ecology and Toxicology Center, 1990). Similarly, other environmental sources such as pharmaceutical products, ambient and indoor air are not normally considered to present a significant source of exposure to N-nitrosamines. N-nitrosamines in ETS (Brunnemann *et al.* 1992; Klus *et al.* 1992; Tricker *et al.* 1994), could in a worst-case situation, result in a maximum total exposure of 20 ng/day TSNA. This corresponds to about 0.1 nmol/day TSNA, or 0.012% of the total daily dietary intake of ATNC.

Based on the above evaluations, total exogenous exposure to N-nitrosamines is estimated to be about 1.10 $\mu\text{mol/day}$; the major exposure sources are the diet (0.79 $\mu\text{mol/day}$; 80–120 $\mu\text{g/day}$; 72%), occupational exposure (0.15–0.30 $\mu\text{mol/day}$; 20–80 $\mu\text{g/day}$; 25%), cigarette smoking (0.02 $\mu\text{mol/day}$; 3.4 $\mu\text{g/day}$; 2%) and miscellaneous minor sources such as pharmaceutical products, cosmetics, indoor and outdoor air (0.001 $\mu\text{mol/day}$; 0.1 $\mu\text{g/day}$; 1%). This assessment is significantly different to that of the American National Academy of Sciences, who in 1981, assessed the main sources of exposure to be the diet (0.3–20.0 $\mu\text{g/day}$), cigarette smoking (17 $\mu\text{g/day}$), cosmetics (0.4 $\mu\text{g/day}$) and occupational exposure (10–180 $\mu\text{g/day}$) (National Academy of Sciences, 1981).

With the exception of certain NVNA, most N-nitrosamines to which man is exposed are only partially excreted in urine, if at all, and the extent of their endogenous formation remains unknown. The excretion of NVNA observed in some Chinese areas with a high prevalence of oesophageal cancer, although generally elevated compared with the respective areas with low prevalence rates (Lu *et al.* 1986, 1987), are no higher than reported in normal healthy subjects in (West) Germany (Tricker *et al.* 1989b), Japan (Tsuda *et al.* 1987) or the UK (Tricker, 1996). Conversely, healthy Egyptian subjects (Tricker *et al.* 1989b) have a considerably higher NVNA excretion than subjects living in high-prevalence areas for gastric cancer in Poland (Zatonski

et al., 1989) and Japan (Kamiyama *et al.*, 1987), or populations with a high-risk for oesophageal cancer in Japan (Tsugane *et al.*, 1992) and China (Lu *et al.*, 1986, 1987). These studies reflect both regional differences in the incidence and pattern of occurrence of dietary *N*-nitrosamines (Gao *et al.*, 1991) and large interindividual variations in excretion (Garland *et al.*, 1986; Tannenbaum, 1986) which may not necessarily be related to cancer risk in different geographical areas.

Determining NPRO excretion after administration of proline (NPRO test) as an index of endogenous nitrosation has several inherent weaknesses, even when corrections are made for the excretion of dietary NPRO intake (Licht and Dean, 1988; Shuker and Forman, 1991). Proline is only nitrosated under a narrow acidic pH range (pH 1.0–3.0), somewhat lower than for most amines (Mirvish, 1975), is not nitrosated by bacteria (Calmels *et al.*, 1985), and is a poor substrate for cell-mediated nitrosation (Miwa *et al.*, 1989). As a consequence, the NPRO test shows reduced NPRO formation in achlorhydric subjects in contrast to the gastric cancer hypothesis (Correa *et al.*, 1975) (Table 4). These inherent weaknesses may also explain why the NPRO test produces inconsistent and contradictory results in population studies (Table 5) and in comparisons of smokers and nonsmokers (Table 6). The NPRO test also indicates that consumption of betel with and without tobacco increases the potential for endogenous *N*-nitrosation (Nair *et al.*, 1986; Chakradeo *et al.*, 1994), although no conclusive evidence is available to support an increase in urinary NVNA excretion (Nair *et al.*, 1985, 1986; Chakradeo *et al.*, 1994). Contrary to this, chewing tobacco does not increase (and probably inhibits) *in vivo* nitrosation (Mirvish *et al.*, 1995). From these studies it can only be concluded that the various NPRO test protocols (Bartsch *et al.*, 1990) require further development if possible confounding factors are to be avoided and meaningful results obtained (Mirvish *et al.*, 1995).

The endogenous nitrosation of pharmaceutical products containing piperazine is probably the best example currently available to show the potential for endogenous *N*-nitrosation in man (Bellander, 1990; Tricker *et al.*, 1991d; Kumar *et al.*, 1992). Endogenous nitrosation of a therapeutic dose of piperazine (2 g/day) can result in endogenous formation and urinary excretion of 0.6–105 µg/day (0.005–0.90 µmol/day) *N*-nitrosamines. However, the actual extent of endogenous nitrosation is probably very much higher (Bellander, 1990). Under normal conditions, high gastric concentrations of

other readily nitrosatable amine precursors would also be expected to result in significant levels of endogenous *N*-nitrosamine formation.

Continuous endogenous formation of *N*-nitrosamine is evident in both experimental animals (Van Stee *et al.*, 1995) and in man (Dunn *et al.*, 1990). However, the full extent of bacterial and cell-mediated nitrosation is impossible to evaluate. Increased bacterial nitrosation occurs in the achlorhydric stomach (Tables 7 and 8), and in patients with infections of the urinary tract (Ohshima *et al.*, 1987; Stickler *et al.*, 1992) or urinary diversions (Tricker *et al.*, 1989c; Gröschel *et al.*, 1992). Increased cell-mediated NO production by blood mononuclear leukocytes in alcoholic cirrhosis (Ciriado-Jiménez *et al.*, 1995) may be related to the increased excretion of NVNA (Habib *et al.*, 1986; Bartsch *et al.*, 1990). Inflammatory cell infiltration in the liver (Paronetto and Tennant, 1990) and immunostimulation of hepatocytes (Curran *et al.*, 1989), macrophages (Marletta *et al.*, 1988) and Kupffer cells (Biliar *et al.*, 1989) may also contribute to the increased *N*-nitrosamine excretion in subjects infected by *O. viverrini* (Srianujata *et al.*, 1987) and *S. mansoni* (Mostaffa *et al.*, 1994). Increased endogenous *N*-nitrosamine formation may be a byproduct of increased NO synthesis, a general response involved in defence against infecting organisms and inflammation (Lui and Hotchkiss, 1995).

It is also speculated that bacterial nitrosation occurs in the nasopharynx (Charrière *et al.*, 1991) and vaginal vault (Pavic, 1984). Similarly, *Candida albicans* and other yeasts (*C. tropicalis*, *C. parapsilosis* and *Torulopsis glabrata*) may increase the potential for endogenous *N*-nitrosamine formation in the oral cavity of subjects with poor oral hygiene (Krogh *et al.*, 1987). Although not extensively studied, NDMA has been reported in breast milk (Mizuishi *et al.*, 1987; Uibu *et al.*, 1996) and in pooled vaginal exudate from patients with *Trichomonas vaginalis* (Harington *et al.*, 1973), and NPRO in sweat (Bogovski *et al.*, 1984).

Nothing is currently known about the extent of dietary ATNC absorption in the gastrointestinal tract and excretion in urine and faeces. Gastric ATNC are elevated in subjects with CAG and achlorhydria (Pignatelli *et al.*, 1987, 1991; Xu and Reed, 1993b,c; Reed *et al.*, 1996). Further evidence suggests that endogenous formation of ATNC also occurs in the intestine (Massey *et al.*, 1988); both nitrate and nitrite are formed endogenously in the human intestine (Tannenbaum *et al.*, 1978), and several strains of intestinal bacteria can catalyse

N-nitrosation (Suzuki and Mitsuoka, 1984). Total faecal ATNC excretion is reported to be 1.56 ± 1.56 (range 0.45–5.33) $\mu\text{mol/day}$ ATNC (Rowland *et al.*, 1991). Assuming that an adult has an excretion of 300 g faeces/day, a total faecal excretion of 3.17 ± 2.58 (range 0.39–9.93) $\mu\text{mol/day}$ ATNC can be calculated from another study (Pignatelli *et al.*, 1990). VNA are not present in faeces (Lee *et al.*, 1981); however, the presence of NVNA cannot be excluded (Ohshima *et al.*, 1982; Van Broekhoven *et al.*, 1989; Tricker *et al.*, 1993).

Mean urinary excretion of ATNC amounts to 1.00 ± 0.81 (range 0.2–3.2) $\mu\text{mol/l}$ urine (Pignatelli *et al.*, 1989). Assuming that an adult has a mean urinary excretion of 1.3 l/day, a total urinary excretion of 1.30 ± 1.05 $\mu\text{mol/day}$ ATNC can be estimated. Excretion of NVNA (NSAR, NPRO, NTCA and NMTCa) accounts for about 16% of the ATNC response (Pignatelli *et al.*, 1989). From other studies the contribution of NDMA (Garland *et al.*, 1986) and NHPRO (Tricker and Preussmann, 1987) to the urinary ATNC response can be calculated to be about 0.03% and 0.6%, respectively. In cigarette smokers total NNAL and NNAL-Gluc excretion (Table 3) would account for about 0.2% of the ATNC response, or <1.5% of the total identified N-nitroso compounds in urine. In subjects with bacterial infection of the urinary bladder the excretion of urinary ATNC is increased (Hicks *et al.*, 1982; Ohshima *et al.*, 1987).

A crude mass balance between total exogenous exposure to N-nitrosamines (1.10 $\mu\text{mol/day}$) and ATNC excretion (faeces, 1.56 ± 1.56 to 3.17 ± 2.58 $\mu\text{mol/day}$; urine, 1.30 ± 1.05 $\mu\text{mol/day}$) indicates that about 40–75% of the total human exposure to N-nitroso compounds results from *in vivo* formation. However, the validity of this estimate is limited, since dietary intake, accounting for 72% of the total exogenous exposure, and faecal ATNC excretion were determined by the same working group using the Walters method, while urinary ATNC was calculated from data obtained using the Pignatelli method. Nevertheless, a crude mass balance based only on data obtained for ATNC using the Walters method (i.e. dietary intake and faecal excretion) indicates that *in vivo* formation of N-nitroso compounds accounts for at least 50% of the total human exposure. Thus, confirmation is provided that endogenous formation of N-nitroso compounds could provide the largest source of exposure for the general population (National Academy of Sciences, 1978).

In light of the above comments it would appear that suitable biomonitoring methods to

detect human exposure to the vast majority of N-nitrosamines are still not available, and basic research has often focused on identifying and developing methods for determining minor sources of exposure. It still remains to be established whether exogenous exposure to preformed N-nitrosamines and endogenous nitrosation of secondary amines or primary amines to form DNA-reactive species (Huber and Lutz, 1984; Shuker and Bartsch, 1994) are of secondary biologic importance to reactions of endogenous NO with intracellular and extracellular macromolecules (Lui and Hotchkiss, 1995). Reactions of NO to form S-nitrosothiol adducts appears to inhibit the activity of thiol-containing enzymes (Laval and Wink, 1994; Wink and Laval, 1994), indicating a dual role for endogenous NO synthesis in inducing mutation: endogenous N-nitrosamine formation and inhibition of repair of lesions which may be induced by these compounds. Despite intensive investigation, no human cancer has been shown to be the result of exposure to preformed N-nitroso compounds in the diet, tobacco, tobacco smoke and other occupation sources, or to such compounds formed *in vivo*. Although several independent pieces of circumstantial evidence support the role of N-nitrosamines in the aetiology of some cancers, a direct link between exposure to exogenous or endogenous N-nitrosamines may never be possible (Mirvish, 1995).

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